STUDIES ON IMMOBILIZED PAPAIN USING POLYCLONAL ANTIBODIES

THESIS

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My Parents

(for leading me to intellectual horizons)

Fakhrejahan

(for her unending support)

Shadab

(for making it worthwhile)

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CERTIFICATE

I certify that the work presented in this thesis entitled **'Studies on Immobilized Papain Using Polyclonal Antibodies'** has been carried out by ***Shakil Ahmad Khan*** under my supervision. It is original in nature and has not been submitted for any other degree.

Jawaid

(JAWAID IQBAL)
Ph.D.

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Shakil A. Khan.

Date : 17th August, 1998

(SHAKIL AHMAD KHAN)

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LIST OF ABBREVIATIONS

Ag	Antigen
BAPNA	Benzoyl-DL-arginine-p-nitroanilide
BSA	Bovine serum albumin
DEAE	Diethyl aminoethyl cellulose
DTNB	5,5' Dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
IgG	Immunoglobulin G
M	Molar
PAGE	Polyacrylamide gel electrophoresis
SH	Sulfhydryl
SDS	Sodium dodecyl sulphate
Immobilized Preparations	
A	'Low' antibody/enzyme ratio
B	'High' antibody/enzyme ratio
H	'High' papain concentration used for coupling with Seralose-4B
H_1	'High' papain concentration coupled to antiserum bound Seralose-4B
H_2	'High' papain concentration coupled to IgG bound Seralose-4B
L	'Low' papain concentration used for coupling with Seralose-4B
L_1	'Low' papain concentration coupled to antiserum bound Seralose-4B
L_2	'Low' papain concentration coupled to IgG bound Seralose-4B

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INTRODUCTION

GENERAL

Enzymes are biological catalysts that regulate the multitude of chemical reactions that occur in the living cell. They mediate such cellular processes as energy conversion, food digestion and biosynthesis. Microbial enzyme preparations have been used in the production of numerous food products for years. Their practical applications in fermented products such as cheese, bread and alcoholic beverages dates back many centuries, long before the nature and function of enzymes or even the microorganisms themselves, were known or understood. With the understanding of the nature of enzymes and their catalytic potency, industry began exploiting these versatile agents. Enzymes isolated from their parent organisms have had a long history of commercial use in pharmaceutical industries as well as in food production (Cheetham, 1985; Arbige and Picher, 1989; Barker, 1988). Now the challenge lies elsewhere as well, including the biosensors which are employing widespread applications in analytical and biomedical fields (Williams and Blanch, 1994; Hoshi *et al.*, 1995; Wang *et al.*, 1995).

Inspite of these advantages, the use of enzymes in industrial applications have been so far limited by several factors. These include the high cost of enzyme isolation and purification, limited stability especially for those enzymes which are unstable when removed from the living cells, poor recovery from reactor effluents at the end of catalytic process and limited reusability. On

the other hand the practical use of enzymes often requires working denaturating conditions such as (i) elevated temperature to increase productivity and prevent microbial contamination, (ii) aqueous-organic environment to shift the reaction equilibrium towards the desired product, and (iii) reaction pHs different from those where enzymes show the maximum pH stability. In order to overcome these problems and to develop reusable catalysts suited for analytical, biomedical and industrial applications, immobilization technology has been considered an excellent approach to stabilize enzymes.

ENZYME IMMOBILIZATION

The immobilization of enzymes is a technique widely employed both in fundamental studies in biochemistry and in practical applications in biotechnology. By definition, an immobilized enzyme is a protein physically localized in a certain region of space or converted from a water soluble mobile state to a water insoluble immobile one (Marconi, 1984). Biotechnological applications of immobilized biocatalysts include several fields of general interests and in particular, clinical and analytical chemistry, medicine, food and pharmaceutical technology, organic synthesis and industrial production of chemical compounds (Clark, 1994; Phadke, 1995; Lebedeva *et al.*, 1991). The success of enzyme immobilization technology is dependent on the choice of carrier and method of immobilization. Several organic, inorganic and synthetic

polymeric materials have been used as support for the immobilization of enzymes (Wehtje *et al.*, 1993; Chaga, 1994; Orberger *et al.*, 1993; Husain *et al.*, 1992; Liao and Lee, 1997; Fachsbauer *et al.*, 1996).

Several methods for the immobilization of enzymes have been developed in the last few decades. These techniques can be broadly classified into the following types (a) physical adsorption, (b) covalent coupling, (c) crosslinking or chemical aggregation, (d) entrapment, and (e) microencapsulation.

(a) Physical adsorption

The oldest method of enzyme immobilization is physically adsorbing the enzyme onto the solid support that has not been specifically functionalized for covalent attachment. The adsorption process involves ionic, hydrophobic, hydrogen bonds or Vander Wall's interactions which are noncovalent in nature. The reversability of adsorption may be an advantage since intact recovery of both enzyme and carrier may be possible. Cellulose based ion-exchange resins (e.g. carboxymethyl cellulose and DEAE cellulose) have been extensively used. Other materials used for immobilization include polystyrene resins, activated charcoal, collagen, alumina, silica gel and glass (Ison *et al.*, 1990; Ma *et al.*, 1996; Batra and Gupta, 1994).

(b) Covalent coupling

This is the most widely used method and involves covalent coupling of an enzyme to a solid support. This method is advantageous over adsorption as the risk of enzyme desorption during operation is minimized. Among the most common forms of activated polymer are hydrogels like cellulose or polyacrylamide onto which diazo, carbodiimide or azide groups have been incorporated. Hydrogels can be activated directly using cyanogen bromide. Other supports generally used for covalent immobilization are agarose, dextrans, starch, Sephadex, nylon, alumina, carbon and silica (Bhardwaj *et al.*, 1992; Guisan *et al.*, 1993; Liao and Lee, 1997).

(c) Crosslinking or chemical aggregation

Crosslinking of enzymes can be achieved with the help of multifunctional reagents. These reagents can be used not only to link enzymes to cellulose or other polymers, but also to link enzyme molecules to each other. The most commonly used multifunctional reagent is glutaraldehyde. The bifunctional or multifunctional reagents either polymerize or precipitate the enzyme to produce an insoluble complex. Other multifunctional reagents used are 2,4 dinitro -3, 5 diflourobenzene and N,N'-bisdiazobenzidine -2,2'-disulphonic acid, trichlorotriazine and hexamethylene diisocyanate (Zhuang and Butterfield, 1992; Manonmani and Joseph, 1993).

(d) Entrapment

Several reports describing the immobilization of enzymes by entrapment in polymeric matrices are available (Petach and Driscoll, 1994; Guyomard *et al.*, 1996; Fachsbauer *et al.*, 1996). The entrapment procedures are generally not suitable for enzymes acting on large molecular weight substrates. Entrapment is achieved by the formation of a crosslinked polymeric network around the enzyme molecule. The most commonly used polymeric system is polyacrylamide, although alginate, silica, starch, polyamide, silicon and rubber have also been used (Khare *et al.*, 1994).

(e) Microencapsulation

This can be achieved by coacervation (a physical phenomenon) or by interfacial polymerization (a chemical process). This method of immobilization is suitable for enzymes acting on low molecular weight substrates. The significance of encapsulation is that enzymes remain chemically unmodified and hence usually catalytically active. Microencapsulation of enzymes provide large surface area for the accessibility of substrates and catalysts within a relatively small volume. This method also allows simultaneous immobilization of many enzymes in a single step. Enzyme immobilization by entrapment in microcapsules was first reported by Chang (1964) and subsequently persued by others (Parthasarathy *et al.*, 1994; Boivin *et al.*, 1991).

PROTEOLYTIC ENZYME IMMOBILIZATION

Proteolytic enzymes play major role in biological processes and in industries. The most widely used proteases in industries are trypsin, α-chymotrypsin, papain, bromelain, ficin, carboxypeptidase A, caldolysin, terrilytin, subtilisin, thermolysin, pepsin, calpain, etc. and several proteinases from thermophylic organisms. Generally all proteases are susceptible to autodigestion. For easy recovery, reuse, and stabilization various attempts have been made to immobilize these proteases with varying success (Chui and Wainer, 1992; Bhardwaj *et al.*, 1992; Guisan *et al.*, 1993; Kulik *et al.*, 1993; Hayashi *et al.*, 1993b; Willner *et al.*, 1993; Orsat *et al.*, 1994; Petach and Driscol, 1994; Kondo *et al.*, 1994).

The best suitability of the method depends on the maximum retention of activity and greater stabilization. Bhardwaj *et al.* (1992) have selected covalent coupling procedure for the immobilization of trypsin and observed significant retention of activity and greater thermostability as compared to the soluble enzyme. The immobilized preparation could be reused several times with limited proteolysis. To stabilize α-chymotrypsin against irreversible thermal inactivation at high temperatures, methods of covalent modification and multipoint immobilization in combination with the addition of salting in compounds were used (Penova *et al.*, 1994). Greater stabilization of

immobilized α-chymotrypsin by salting in media has been achieved only in proteins with minimum number of bonds with the support.

Trypsin was covalently bound to oxirane-acrylic beads by Lorenzen and Schlimme (1995). About 85% immobilization could be achieved after incubation for 48 hrs. The immobilized preparation was more stable and could be used nine times without loss of activity. Holt and Puleo (1996) have investigated the stability and elutability of trypsin immobilized on inorganic biomaterial alloys. Immobilized trypsin gave significantly higher activity and stability compared to free soluble enzyme. Properties of immobilization of terrilytin, trypsin, collytin or protease C to cellulose copolymer were investigated by Virnik *et al.* (1996). They established that the molecule of anti-microbe substance enhanced the stability of the immobilized enzymes. α-chymotrypsin was deposited on a porous support material, celite by Adlercreutz (1993) and the effect of presence of additives polyols and simple saccharides was investigated. Srokov and Ci (1993) have studied the stability of the photochemically immobilized alkaline proteinase and chymotrypsin onto the gel of hydroxyethyl cellulose. The immobilized alkaline proteinase exhibited a decrease in the ability of denaturation and an increased laboratory stability. Wilson *et al.* (1994) have immobilized an extracellular proteinase to controlled pore glass beads with the retention of 65% of its maximum activity against azocasein at pH 12. Stability at 80°C increased on immobilization at all pH values within 5 and 11 and greater increase in the half life was also observed. Sears and Clark (1993)

have immobilized trypsin to porous glass in both the presence and absence of acetylated soyabean trypsin inhibitor and observed the increase in free energy barrier for amide and ester hydrolysis and decrease in the energy barrier for aminolysis.

Chui and Wainer (1992) have entrapped trypsin and α-chymotrypsin in hydrophobic cavities, with the retention of hydrolytic activity and sensitivity to change in temperature. Trypsin has also been immobilized noncovalently on DEAE cellulose after amino group modification (Tyagi et al., 1994). Pyromelliticdianhydride (PMDA) was used as a modifying agent. The pH optima and K_m of the enzyme remained unaltered suggesting the free accessibility of the substrate. Leuba *et al.* (1993) have demonstrated the use of immobilized trypsin and chymotrypsin in the location of linker histones H_1 and H_5 in chicken erythrocyte chromatin.

Markvicheva *et al.* (1994) have developed one-step method for enzyme immobilization on a thermally reversible polymer. After immobilization, carboxypeptidase B and trypsin retained nearly 80% activity and were active in wider pH and temperature range. Immobilization of chymotrypsin and terrilytin on carbon materials was carried out by Sevast'ionova and Davidenko (1993). The kinetics of the proteolytic enzymes were considered and properties of resultant preparations (temperature and pH optima) and the effect of gamma-sterilization were discussed. Belov *et al.* (1994) have found the complex effect

of the bound enzyme concentration on the proteolytic activity of trypsin immobilized to dialdehyde-cellulose after gamma irradiation and in process of storage. The proteolytic activity of samples after gamma irradiation had increased with the increase of carrier enzyme concentration and did not change during the process of storage.

The Michaelis constant of α-chymotrypsin, immobilized on a glutaraldehyde-activated silicate support was determined by Blais and Lortie (1993) and was identical to soluble enzyme. Michaelis constant was extrapolated and the effect of the immobilized enzyme distribution inside the porous matrix was investigated. Conjugates of bovine serum albumin (BSA) and α-chymotrypsin (CHT) with poly(ethylene glycol) and ethylene oxide and propylene oxide (proxanols) were synthesized by Topchieva *et al.* (1995). It was observed that the conjugates retain high enzymatic activity towards the substrates investigated. These conjugates showed higher thermostability. Creagh *et al.* (1993) have studied structural and catalytic properties of α-chymotrypsin and liver alcohol dehydrogenase in reverse-micelle solutions. α-chymotrypsin retained higher activity in reverse micelle and in some cases displays enhanced activity compared to liver alcohol dehydrogenase. Ge and Zhang (1996) have immobilized pancreatic exopeptidases on chitin by crosslinking with glutaraldehyde. The immobilized pancreatic exopeptidases (IPPE) was effective in releasing the free amino acids from peptides.

Several other insoluble carriers for the immobilization of trypsin have been reported (Glassmeyer & Ogle, 1971; Telefoncu, 1983). Insoluble papain, trypsin, chymotrypsin and urease were prepared by using a diazotized copolymer of para-aminophenyl alanine and leucine as an insoluble carrier (Glazer *et al.*, 1962 and Katchalski, 1962).

PAPAIN STRUCTURE AND FUNCTION

Cysteine proteinases, a class of enzymes having reactive residue at the active centre, are abundent in living cells and play important roles in intracellular proteolysis. The imbalance in their enzymatic activities causes serious diseases such as muscular dystrophy (Katunuma and Kominami, 1987), osteoporosis (Delaisse *et al.*, 1984) and tumor invasion (Denhardt *et al.*, 1987).

Papain, a plant endopeptidase is the best characterized member of the cysteine proteinase family (Mellor *et al.*, 1993; Brocklehurst, 1987; *Brocklehurst et al.*, 1987; Polgar, 1990; Varughese *et al.*, 1989; Stubbs *et al.*, 1990; Bjork and Ylinenjarvi, 1990; Menard *et al.*, 1990; Khouri *et al.*, 1991; Harris *et al.*, 1992 and Lindahl *et al.*, 1992). It is an attractive molecule to use in the study as it is easy to work with, its crystallographic structure has been elucidated (Drenth *et al.*, 1970) and it shows a broad range of hydrolytic activity with a wide variety of esters, amide and peptides. Its mechanism of action is similar to the serine proteases except that cysteine replaces serine in the formation of

the acyl intermediate. It was first isolated in the crystalline form from Carica Papaya latex by Balls and Co-workers (1937) and later it was extensively studied and properties were reviewed by Kimmel and Smith (1954). It consists of a single polypeptide chain of 212 residues and molecular weight of 23406 (Fersht, 1985; Baker and Drenth, 1987). Its amino acid sequence was first reported by Light and collaborators (1964); who described both the tentative linear sequence of the 212 amino acids and the assignment of the positions of the disulfide bridges as well as the active sulfhydryl groups in the molecule.

The three dimensional x-ray study of papain revealed that the conformation of the polypeptide chain is irregular with the exception of one short segment of β structure and four short α-helical segments. The shape of the molecule is spheroidal, with dimensions of about 36 x 48 x 36 Å, and the main chain is folded into two distinct parts which are divided by a cleft. The structural conformation is stabilized by three disulfide bridges.

The papain molecule is folded to form two interacting domains. The catalytically important residues Cys^{25} and His^{159} are located at the interface on opposite domains of the molecule, Cys^{25} being a part of the L1 α-helix at the surface of the left domain, while His^{159} is, in a β-sheet of the right domain of the enzyme. $Asparagine^{175}$ residue lies adjacent to the catalytic His^{159} residue (Vernet *et al.*, 1995). At optimal activity pH, Cys^{25} and His^{159} exist as a thiolate-imidazolium ion pair, S^--ImH^+, in equilibrium with SH-Im (Lewis *et al.*, 1981).

The pka of Cys^{25} changes from 7.8 to 4.4 on protonation of His^{159} while the pka of His^{159} shifts from 5.1 to 8.5 on deprotonation of Cys^{25} (Lewis *et al.*, 1976, 1981; Menard *et al.*, 1990). Other residues Gln^{19}, Ser^{176}, Asn^{175} and Asp^{158} have been implicated in catalysis or/and binding (Baker and Drenth, 1987).

Papain requires a free sulfhydryl group for its catalytic activity. In the native crystalline protein the thiol group appears to be blocked mainly in the form of mixed disulfide with half cystine and exhibits extremely low proteolytic activity (Finkle and Smith, 1958; Hill *et al.*, 1959) and a correspondingly low thiol titer. Activation is achieved by mild reducing agents such as cysteine, sulfide, sulfite as well as cyanide. Optimum activation occurs upon simultaneous application of a thiol compound like cysteine or thioglycolate and a heavy metal-binding agent like ethylenediaminetetraacetic acid (EDTA) or by the addition of BAL (2,3 bismercaptopropanol). The standard activation conditions which are used in activity assays of papain require a medium containing 0.05 M cysteine and 0.02 M EDTA.

Papain is reversibly inactivated in the presence of air and low concentrations of cysteine, which is enhanced by Fe^{2+} and Cu^{2+}. In the absence of cysteine, activated papain is inactivated at a much lower rate in an almost irreversible manner. Heavy metal ions such as Cd^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} are inhibitory for papain (Sluyterman, 1967). Cd^{2+} has a strong, Zn^{2+} a very strong preference for inhibitory combination with papain over

binding with cysteine. Fe^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} show no or a small preference for papain over cysteine. All sulfhydryl reagents act as papain inhibitor. Iodoacetic acid and iodoacetamide react with the free sulfhydryl group of papain, causing irreversible inactivation (Shapira and Aronon, 1969; Kimmel *et al.*, 1965). Several structural inhibitors such as carbobenzoxy-L-glutamic acid act in the region of pH 3.9 to 4.5 causing a non-competitive or a mixed type inhibition. Low concentrations of KCNO (0.1 mM) cause inactivation of papain. Papain is noted for its wide specificity regarding both proteins and small molecular weight substrates. Most of the peptide bonds are hydrolyzed by papain and is capable of catalyzing transamidation, transpeptidation as well as transesterification. It degrades protein substrates more extensively than trypsin, pepsin and chymotrypsin. Immunological studies indicate that papain is highly immunogenic in rabbits (Arnon, 1965; Arnon and Shapira, 1967a). The purified specific antibodies react with papain to yield a single precipitation band in agarose gel diffusion, and are usually not directed against the catalytic site of papain (Arnon and Shapira, 1967b).

PAPAIN IMMOBILIZATION

The thiol protease papain has wide industrial and pharmaceutical applications. The major reasons for its immobilization are undoubtedly its practical applications. Papain has great potential during beer finishing operations in the brewery (Wiseman, 1993) and in meat industry for facilitating

the portioning of meat (Swanson *et al.*, 1992). Papain is also used in medicine for the treatment of various diseases (Karski *et al.*, 1994; Theissen *et al.*, 1995; Udod *et al.*, 1987). Due to the limitation of the use of soluble enzyme for such processes, the advantages offered by immobilized enzyme technology have been explored.

Hayashi *et al.* (1993a) have prepared water-insoluble papain by immobilizing this protease onto the porous poly(vinyl alcohol) (PVA) beads by covalent fixation with an increase in K_m and decrease in Vmax value. The pH, thermal and storage stabilities of the immobilized enzyme were higher than the soluble enzyme. Huckel *et al* (1996) have covalently immobilized papain to the surface of a support material porous zirconia and have compared this support with porous activated silica having similar surface area/g support material. Higher amount of papain could be immobilized on the activated zirconia support. The amount of active papain immobilized at pH 9 to the activated silica was lower than achieved with the activated zirconia. Papain was covalently immobilized by Itoyama *et al* (1994) onto the surface of porous chitosan beads without or with spacers of different lengths. The pH, thermal and storage stabilities of the immobilized papain were higher than those of the soluble one. The spacer's effect on the activity of the immobilized papain could be explained in terms of mobility of the immobilized papain molecule. Papain was covalently immobilized onto vinyl alcohol/vinyl butyral copolymer (PMB) membrane (Zhuang and Butterfield, 1993). The immobilized papain bioreactors

showed better storage, reusability and thermal stability as compared to soluble enzyme. The insertion of a 6 carbon spacer between the membrane and covalently bound enzyme reduced the disturbance of the enzyme systems, resulting in K_m values intermediate between the free and directly bound enzyme.

Ogi *et al* (1990) have immobilized papain on amino organosilica and have studied its kinetics as well as physicochemical properties. The pH-optima, pH activity profile and K_m of the enzyme after immobilization remained unchanged, compared to soluble enzyme. After 20 days of storage, the immobilized papain lost about 20% activity. Eckstein *et al* (1991) have studied the immobilization of papain on two commercially available carriers, VA Biosynth and Eupergit C, in their epoxidized form. Papain was immobilized on both polymers and showed high activity. The stability (lyophilization, storage) of the papain-VA Biosynth improved remarkably, and after prolonged storage (-20 degree celcius) it could be reactivated to its full activity. Properties of papain immobilized on viscose fibres were studied by Liubich *et al* (1980) using two spacer arms : maleic dialdehyde and cyanuric chloride. The enzyme thermostability, urea denaturation and resistence to autolysis were examined. Greater denaturation stability was achieved with the use of cyanuric chloride as a spacer arm. Storozhuk *et al* (1985) have immobilized papain on fibre polymer materials which was used in the treatment of acute destructive lactation mastitis.

Ohmori and Yang (1994) have entrapped papain in polyacrylamide gel. Papain was entrapped within a relatively large space of polymer network and the gel matrix was uncharged. They investigated the dynamic behaviours, particularly autonomous pH oscillations of a papain membrane system using the IS-FET (ion-selective field effect transistor) sensor. Papain was non-covalently immobilized onto the poly(ether)sulfone membrane via the avidin-biotin complex (Bhardwaj *et al.*, 1996). Kinetic parameters for the amidase activity of non-covalently bound papain, using the substrate benzoyl arginine p-nitroanilide hydrochloride (BAPNA), were determined and the results were compared with soluble papain as well as papain immobilized directly onto the modified poly(ether)sulfone membrane. A decrease in the enzymatic activity was observed upon direct immobilization. Insertion of avidin-biotin complex to non-covalent spacer increased the apparent Vmax and decreased the K_m relative to directly immobilized papain. The non-covalently attached enzyme-bioreactor showed significant increase in stability and reusability compared to soluble enzyme.

Fukal *et al* (1983) have studied the effect of different chemical modifications of papain on the proteolytic activity. Modification with Dextran T 2000 caused increasing decline in proteolytic activity, while modification of papain with glutaraldehyde and formaldehyde caused rapid drop in activities even at very low concentration. Lozano *et al* (1993) in an another study have

shown the influence of different polyhydroxylic cosolvents containing two to six carbon atoms on the thermostability of papain at 60°C.

Chiou and Beuchat (1986) have immobilized papain on an anion-exchange resin by physical adsorption followed by crosslinking with glutaraldehyde and applied them on continous flow reactor. Activity of immobilized papain increased with increase in temperature from 37°C to 85°C and then decreased at higher temperature. The stability and proteolytic activity of papain were studied by Vicente *et al* (1994) in reverse micellar systems, and in aqueous media. Higher papain activity and stability was found in reverse micellar systems compared with the aqueous solution. Papain was covalently immobilized onto the thermosensitive N-alkyl acrylamide latex particles with high efficiency by the carbodiimide method (Kondo *et al.*, 1994b). The immobilized enzyme showed extremely high activity towards casein and retained the activity without any definite loss during repeated thermocycles.

Kumakura and Kaetsu (1984) have immobilized papain by entrapping it in the matrix formed by radiation polymerization of various monomers at low temperatures, and behaviour of the immobilized enzyme was studied. The enzyme activities upon immobilization increased with increase of the degree of hydration of the polymer. Harhen and Barry (1990) have studied the properties of papain attached to controlled pore glass and Sepharose by coupling with glutaraldehyde and cyanogen bromide respectively. The results indicated that

the enzyme could be successfully immobilized on these supports and its stability to heat, denaturation and storage was maintained or improved.

IMMOBILIZATION ON ANTIBODY SUPPORT

One potential approach in the immobilization of enzymes that does not involve chemical modification and facilitates excellent access of the substrate for bound enzyme is the use of bioaffinity matrices including specific antibodies as affinity supports. Enzymes immobilized on supports precoupled with antibodies exhibit almost full catalytic activity on their respective substrates. Some reports are available on the formation of insoluble antibody-enzyme complexes retaining improved enzyme activity. Burnett and Schmidt (1921) have demonstrated for the first time that insoluble complex of catalase and anticatalase from rabbit serum retained 100% of the original activity. Glucose oxidase (de Elwis *et al.*, 1987), Invertase (Jafri *et al.*, 1993), Transglutaminase (Ikura *et al.*, 1984) and Carboxypeptidase A (Solomon *et al.*, 1986) have been immobilized on antibody supports retaining full catalytic activity.

Sada *et al* (1986) have studied the effect of pH, ionic strength, anion species, and antibody (ligand) concentration on the adsorption equilibrium between the immobilized antibodies and antigens, using anti-bovine serum albumin (BSA), anti-human serum albumin (HSA) and anti-bovine immunoglobulin G (BIgG) coupled to Sepharose-4B. The antibody ligand was sufficiently stable for repeated use. Since the biological interaction between

antigen and antibody is highly specific and strong, the use of immobilized antibodies against these antigens enable them to purify to very high degrees. Solomon *et al* (1986) have investigated the use of monoclonal antibodies in the immobilization of enzymes. The method is based on the binding of enzymes to suitable carriers via monoclonal antibodies that bind to the enzyme with high affinity. These workers have isolated anti-carboxypeptidase A antibodies and immobilized them on Eupergit or Sepharose protein A. The conjugate of antibodies with support were then used for the immobilization of carboxypeptidase A enzyme which was highly active. The antibody component of these immobilized preparations represent an extended spacer separating the enzyme from the carrier. The enzymes immobilized via antibodies would undergo less steric interference by the carrier and thus have more freedom to react even with the high-molecular weight substrates.

Stovickova *et al* (1991) have prepared immobilized trypsin using polyclonal antibodies. The catalytic activity of trypsin was not affected by interaction with these antibodies, even in the presence of excess antibodies. If enzyme escapes from the support or undergoes some irreversible change, it may be easily replaced with active enzyme by additional affinity immobilization. The method represents a general technique suitable for the preparation of highly active immobilized enzyme preparations for the biochemical studies of enzymes naturally bound in the organelle structure, because the kinetics of metabolic processes of an organism and heterogenous catalysis of enzyme

reactors are governed by the same rules. Jafri *et al* (1993) have raised antisera in rabbits against Baker's yeast invertase which significantly activated the enzyme in vitro. The antisera contained precipitating antibodies which appeared to be directed against the glycosyl residues of the enzymes. Invertase could be immobilized as insoluble enzyme antibody adducts or by binding to a Sepharose matrix precoupled with gamma globulin fraction derived from the antisera. The immobilized invertase exhibited high enzyme activity and had markedly enhanced thermal stability, which could be further improved by cross-linking with glutaraldehyde.

Gorvits *et al* (1993) have chosen a two-step immobilization procedure as a method for the immobilization of immunoglobulins, to control the quantity of immobilized protein and also to create a number of variably activated zones on the matrix. Rabbit anti-horseradish peroxidase (HRP) antibodies were photoimmobili-zed on the porous membrane supports, and antigen solution were leaked along the surface of the membrane with immobilized antibodies placed in a flow cell. The binding between the antigen and the antibodies photoimmobilized on the membrane were studied kinetically.

Catalase was non-covalently immobilized on an immunosorbent prepared by anticatalase adsorption on an activated carbon fabric (ACF) and its kinetic parameters were determined (Litvinchuk *et al.*, 1994). Under the optimum conditions the immobilized catalase activity was 1.5 fold higher as

compared to soluble catalase. Antibodies stabilized soluble catalase, but decreased its thermo-stability on immobilization of immunocomplexes on ACF.

A novel use of polyclonal antibodies in the stabilization of enzymes has been studied by Sato and Walton (1983) in which gluconolactone-antibody complex after treatment with glutaraldehyde was administered to guinea pig and synthesis of ascorbic acid was monitered. Nolan and Kennedy (1990) have used bifunctional antibodies in the immobilization of enzymes of specific surfaces. Catalase and acetylcholinesterase were also stabilized by complex formation with their antibodies (Feinstein *et al.*, 1971; Michaeli *et al.*, 1969). These preparations did not affect the enzyme activity nor the K_m value or substrate specificity. Acetylcholinesterase lost its activity upon heating at 60°C for a period as short as five minutes, while the enzyme in the complex with the antibody exhibited remarkable stability at this temperature for a period tested upto 90 minutes. The affinity of enzymes for their antibodies was very strong which could be retained for prolonged durations and improved their thermostability, and resistance to various denaturants.

EXPERIMENTAL

MATERIALS

The chemicals used in the present study were obtained from various sources as shown below. Glass distilled water was used in all the experiments.

Chemical	Source
Acetonitrile	Veb. Lab., Germany
Acetic acid	Sisco Research Lab, India
Acrylamide	Sisco Research Lab., India
Agarose	Sisco Research Lab., India
Ammonium per sulphate	Sisco Research Lab., India
Benzoyl-DL-Arginine-P Nitroanilide (BAPNA)	Sigma Chemical Co., USA
Bovine serum albumin	Sigma Chemical Co., USA
Bromophenol blue	B.D.H. Poole, England
Casein	Central Drug House, India
α-Chymotrypsin	Sisco Research Lab, India
Coomasie brilliant blue	Sigma Chemical Co., USA
Copper sulphate	Sisco Research Lab, India
Cyanogen bromide	Sisco Research Lab, India
L-Cysteine hydrochloride	Sisco Research Lab, India
Cytochrome C	Sisco Research Lab, India
Dextran blue	Pharmacia Fine Chem..Sweden
Diethylaminoethyl cellulose	Sisco Research Lab, India
Dithionitrobenzoic acid (DTNB)	Sisco Research Lab, India

Ethanolamine	Sisco Research Lab, India
Ethylenediaminetetraacetic acid (EDTA)	Hi-Media Lab, India
Folin's Reagent	Sisco Research Lab, India
Formaldehyde	S.D. Fine, India
Freund's complete adjuvant	Difco Laboratories, USA
Freund's incomplete adjuvant	Difco Laboratories, USA
Glutathione	Sisco Research Lab, India
Glycerol	Sisco Research Lab, India
Glycine	Sisco Research Lab, India
Iodoacetamide	Sigma Chemical Co., USA
Iodoacetate	Sigma Chemical Co., USA
β-Mercaptoethanol	Qualigens Fine Chem., India
Methanol	Qualigens Fine Chem., India
N,N, Methylene bis acrylamide	Sisco Research Lab, India
Nitric acid	Sisco Research Lab, India
Papain	Sigma Chemical Co., USA
Potassium dichromate	B.D.H., India
Potassium phosphate	Qualigens Fine Chem., India
Ribonuclease A	Sisco Research Lab, India
Sephadex G-100	Sigma Chemical Co., USA
Seralose-4B	Sisco Research Lab, India
Silver nitrate	E. Merck, India

Sodium carbonate	E. Merck, India
Sodium chloride	E. Merck, India
Sodium Lauryl sulphate	Sigma Chemical Co., USA
Sodium potassium tartarate	Sisco Research Lab, India
TEMED	Sisco Research Lab, India
Trichloro acetic acid	Qualigens Fine Chem., India
Tris (hydroxymethylaminoethane)	Qualigens Fine Chem., India
Urea	E. Merck India

METHODS

IMMUNOLOGICAL STUDIES

1. Preparation of Antigens

i. **Native papain :** 600 μg protein/0.5 ml phosphate buffer was thoroughly mixed with 0.5 ml Freund's complete adjuvant as described by Freund (1947).

ii. **Iodoacetamide treated papain :** Papain was modified by the procedure of Crestfield *et al* (1963). Native papain (3 mg protein/ml) dissolved in 0.5 M Tris buffer, pH 8.6 was incubated in dark for 1 hr at room temperature with 0.3 ml EDTA (50 mg/ml), 0.03 ml, β-mercaptoethanol and 0.7 ml iodoacetamide (50 mg/ml), and finally it was precipitated with saturating concentration of 0-80% ammonium sulphate at 4°C for 12 hrs to concentrate the protein. The precipitate was dissolved in 6 ml, 10 mM potassium phosphate buffer pH 8.6, and dialysed overnight at 4°C to remove ammonium sulphate, and β-mercaptoethanol. 600 μg of protein/0.5 ml of 10 mM phosphate buffer pH 8.6 was mixed with 0.5 ml Freund's adjuvant and thoroughly emulsified.

iii. **Iodoacetic acid treated papain :** Modification of papain was performed by the described procedure of Crestfield *et al* (1963). Native papain (3 mg protein/ml) dissolved in 0.5 M Tris buffer pH 8.6 was incubated in dark for 1

hr at room temperature with 0.3 ml EDTA (50 mg/ml), 0.03 ml β-mercapto-ethanol, and 0.7 ml iodoacetic acid (50 mg/ml). Finally it was precipitated with saturating concentration of 0-80% ammonium sulphate at 4°C for 12 hrs. The precipitate was dissolved in 6 ml, 10 mM potassium phosphate buffer pH 8.6, and dialyzed overnight at 4°C to remove ammonium sulphate and β-mercaptoethanol. 600 μg protein/0.5 ml in 10 mM phosphate buffer pH 8.6 was mixed with 0.5 ml Freund's complete adjuvant and thoroughly emulsified.

2. Immunization

Albino rabbits weighing between 1.5 to 2.0 kg were used. Prior to immunization the rabbits were bled for obtaining serum that served as control in the studies. 300 μg of antigen (native papain, iodoacetamide as well as iodoacetate treated papain) in 0.25 ml, 10 mM phosphate buffer pH 8.6 were mixed with 0.25 ml Freund's complete adjuvant to form a thorough emulsion. These preparations were injected intramuscularly initially at weekly schedule. A booster dose of 150 μg antigen in 0.25 ml, 10 mM phosphate buffer pH 8.6, was mixed with equal volume of Freund's incomplete adjuvant. Before each booster the animals were bled through ear veins to check the antibody titre.

The blood was collected, kept at 4°C for 5 hrs for clot formation. Serum was collected by centrifugation at 2000 rpm for 10 minutes and stored at -10°C.

3. Ouchterlony double diffusion

The precipitation reaction in agarose gels was performed according to the method of Ouchterlony (1949). One percent (1%) molten agarose in 0.9% NaCl containing 0.1% sodium azide was poured on the petriplate and allowed to solidify at room temperature. Required number of wells were punched. 10-40 µl of antisera was added in the peripheral wells, and 40 µl antigen in the central well. The petriplate was incubated at 37°C for 4 hours and then at 4°C over night in order to get a clear precipitin band.

4. Direct binding ELISA

Antibodies against native papain, iodoacetamide and iodoacetic acid treated papain were detected and quantitated by ELISA using polystyrene microwell modules as solid support (Ali *et al.*, 1985).

The test wells were coated with 100 µl antigen (50 µg protein/ml in bicarbonate buffer) incubated for two hours at room temperature and at 4°C for 12 hrs. The antigen coated wells were washed thrice with TBS-Tween (TBS-T) to remove the unbound antigen. The unoccupied sites were blocked with 150 µl of 1.5% BSA in TBS for 6 hrs at room temperature. BSA in TBS was added both in antigen coated wells and control wells. The plates were washed once with TBS-T and serially diluted antibodies (dilution in TBS buffer) were added. After incubating the plates for two hours at room temperature and 12 hrs at

4°C, the bound antibodies were assayed by anti-rabbit-alkaline phosphatase conjugate using p-nitrophenyl phosphate (PNPP) as colorigenic substrate. The plates were incubated at 37°C for 10 minutes and the reaction was stopped by adding 100 µl of 3 M NaOH solution in each well. The absorbance of each well was monitored at 410 nm in the ELISA Reader.

GEL FILTRATION CHROMATOGRAPHY

A column of Sephadex G-100 was prepared as recommended by Peterson and Saber (1962) at room temperature. Sephadex G-100 was allowed to swell in a suitable amount of distilled water for 5 hours in a boiling waterbath. A previously cleaned glass column was mounted vertically and glasswool plugged at the bottom of the column. The column was filled to one third of its length with the operating 10 mM phosphate buffer pH 8.6. The deareated gel slurry was then gently poured into the column with the help of a glass rod. The column was left standing overnight. Flow rate was subsequently increased gradually with the help of stop cock. After accomplishing a constant rate of flow higher than required for final elution, the column was adjusted to the flow rate of 1 drop per 11 sec. The column was thoroughly washed with two bed volumes of operating buffer (0.01 M potassium-phosphate buffer, pH 8.6). In order to determine the uniform packing and void volume (Vo) of the column, 0.02% (w/v) blue dextran was passed through the column. The buffer was carefully removed from the surface and protein sample was applied. The volume of blue dextran or protein solution applied on the column was not more

than 2-3% of the total bed volume of the column. Marker proteins α-chymotrypsin, bovine serum albumin, ribonuclease A and cytochrome C were passed for the determination of Stoke's radius of native papain, iodoacetamide and iodoacetic acid treated papain according to the method of Laurent and Killander (1964).

GEL ELECTROPHORESIS

a) **Polyacrylamide gel electrophoresis (PAGE) :** PAGE was performed according to the procedure described by Laemmli (1970) using a slab gel apparatus manufactured by Atto Co., Japan. A stock solution of 30% acrylamide containing 0.8% bisacrylamide, 1.0 M Tris-HCl (pH 8.8) was prepared and mixed in appropriate order to give a desired percentage of acrylamide; and was then polymerized after inserting a comb that provided template for number of required wells. Bubbles and leaks were avoided. Protein samples prepared in 10% glycerol, Tris buffer of pH 6.8, and traces of bromophenol blue were loaded on the polymerized gel. Running buffer was 0.025 M Tris, 0.2 M glycine, and voltage applied was 100 V.

b) **SDS-polyacrylamide gel electrophoresis :** Tris glycine system of Laemmli (1970) was followed using mini slab gel apparatus manufactured by Atto Co., Japan. Stock solutions of 30% acrylamide containing 0.8% bisacrylamide, 1.5 M Tris-HCl (pH 8.8) and 10% SDS were prepared and mixed in specific proportion to give desired percentage of acrylamide. The

cocktail was poured between glass plates, and comb was inserted for making wells and was allowed to polymerize within 30 minutes. Protein samples were prepared to give a final concentration of 1% SDS (w/v), 0.5% β-merceptoethanol, 0.25 M Tris HCl pH 6.8, 10% (w/v) glycerol and trace of bromophenol blue as a tracking dye. Samples were then treated in a boiling water bath for 3 min. and were applied to the wells. Electrophoresis was carried out at 100 V for approximately two bours in Tris-glycine buffer containing 0.025 M Tris HCl, 0.2 M glycine and 0.2% SDS. Protein bands were detected by staining with silver nitrate method of Merril *et al* (1982).

c) **Silver nitrate staining :** After electrophoresis the gels were immersed in a mixture of 40% methanol and 10% acetic acid for 1 hour with constant shaking for fixation. The gel was washed twice with mixture of 10% methanol and 5% acetic acid, each washing being of 15 minutes to allow the gel to swell to normal size.

The gel was then soaked for 15 minutes in 3.4 mM potassium dichromate solution containing 3.2 mM nitric acid, and washed with distilled water, and again soaked in silver nitrate (12 mM) solution for 20 minutes, with regular shaking. After washing with distilled water, the gel was transferred to 280 mM solution of sodium carbonate containing formaldehyde to make the gel alkaline. After 10 minutes the reaction was stopped by suspending the gel in 3% acetic acid solution for 5 minutes and washed 4-5 times with distilled water.

d) **Coomasie brilliant blue staining :** After completion of electrophoresis the gel was stained for protein with coomasie brilliant blue R-250 in 40% methanol and 10% acetic acid for 4 hrs. The gel was destained for several hours with 10% glacial acetic acid until the blue background became clear.

ISOLATION OF GAMMA GLOBULIN FROM IMMUNE SERA

The gamma globulin (IgG) fraction of the antipapain antiserum was precipitated with $(NH_4)_2SO_4$ and fractions between 20-40% saturations were collected. The precipitate dissolved in minimum volume of phosphate buffer was extensively dialysed against 10 mM phosphate buffer pH 8.6. About 300 mg of ammonium sulphate cut precipitate was added to 5 gm of regenerated DEAE cellulose (Whatman 52) and stirred for 2 hrs at 4°C. The supernatant obtained after centrifugation contained pure IgG which was concentrated before use.

PREPARATION OF INSOLUBLE IMMUNOCOMPLEX OF PAPAIN

Two preparations of papain have been selected for insolubilization. These were designated as Preparations (A) and (B) having low and high antibody to enzyme ratios respectively. For preparation (A) 69.6 mg, while for (B) 4.4 mg of papain was selected. Insoluble papain-antipapain adducts were prepared by adding equal volume of adequate antiserum (78.8 mg/ml). The

tubes were incubated at 37°C for 1 hr followed by 24 hrs incubation at 4°C prior to isolation of precipitate by centrifugation. The precipitate was washed extensively with buffer. The adducts were suspended and stored in the same buffer at 4°C.

IgGs were precipitated from the immune sera with $(NH_4)_2SO_4$ and fractions between 20-40% saturations were collected. The precipitate dissolved in minimum amount of buffer was extensively dialyzed against 10 mM phosphate buffer pH 8.6 and similar adducts were prepared with the IgG by taking the above two concentrations of papain indicated as (A) and (B).

IMMOBILIZATION OF ENZYMES ON IMMUNOAFFINITY SUPPORTS

(a) Coupling of antiserum and IgG to Seralose-4B

Seralose-4B was activated by the procedure of Porath *et al* (1967). Five grams of Seralose-4B was washed thoroughly with distilled water using a glass sintered funnel. The gel was sucked dry and suspended in 5.0 ml distilled water and 5.0 ml of 2.0 M sodium carbonate and mixed thoroughly. One gram cyanogen bromide dissolved in 1.0 ml acetonitrile was added to the beaker containing Seralose-4B and mixed thoroughly at 4°C for 10 minutes. The whole mass was then transferred immediately to a glass sintered funnel and washed thoroughly with 0.1 M bicarbonate buffer pH 8.5, distilled water and once again with the same bicarbonate buffer. The washed activated Seralose-4B was dried and resuspended in 5.0 ml of 0.1 M bicarbonate buffer, pH 8.5.

The activated Seralose-4B was incubated separately with antiserum/ purified immunoglobulin-G (25 mg Protein) at 4°C for 24 hrs. The matrices were thoroughly washed and resuspended in 10 mM phosphate buffer pH 8.6. Amount of protein immobilized was calculated by substracting the protein remaining in supernatent and washing from the amount added to the activated Seralose-4B matrix.

(b) Enzyme immobilization on antibody support

Each gram of the appropriate IgG or antiserum bound Seralose matrix was incubated with 29.0 mg of papain designated as 'high' papain concentration coupled to antiserum bound Seralose-4B (H_1) or IgG bound Seralose-4B (H_2) and 1.85 mg of papain designated as 'low' papain concentration coupled to antiserum bound Seralose-4B (L_1) or IgG bound Seralose-4B (L_2). The preparations were incubated at 4°C for 12 hrs and washed extensively with 10 mM phosphate buffer pH 8.6 and suspended in the same washing buffer.

IMMOBILIZATION OF PAPAIN ON ACTIVATED SERALOSE-4B

Seralose-4B matrix was activated according to the method of Porath *et al* (1967). Five grams of Seralose-4B was washed thoroughly with distilled water using a glass sintered funnel. The dried gel was suspended in 5.0 ml distilled water and 5.0 ml of 2.0 M sodium carbonate and mixed thoroughly.

One gram of cyanogen bromide dissolved in 1.0 ml acetonitrile was added to the beaker containing Seralose-4B and mixed thoroughly at 4°C for 10 minutes. The whole mass was then transferred immediately to a glass sintered funnel and washed thoroughly with 0.1 M bicarbonate buffer pH 8.5, distilled water and once again with the same bicarbonate buffer. The washed activated Seralose-4B was dried and resuspended in 5.0 ml of 0.1 M bicarbonate buffer, pH 8.5.

The activated Seralose-4B was incubated with 25.0 mg papain designated as 'High' enzyme concentration (H) or 1.6 mg papain designated as 'Low' enzyme concentration (L) for 24 hrs at 4°C. The matrices were thoroughly washed and resuspended in 10 mM phosphate buffer pH 8.6. Amount of protein immobilized was calculated by substracting the protein remaining in the supernatant and washing from the amount added to the activated Seralose-4B matrix.

COLORIMETRIC ANALYSIS

Protein Estimation

The procedure described by Lowry *et al* (1951) was followed. Suitable aliquot of the protein sample was diluted to 1.0 ml with distilled water. To this 5.0 ml of freshly prepared alkaline copper reagent was added which was prepared by mixing 0.5% (w/v) copper sulphate in 1% (w/v) sodium potassium tartarate and 2% (w/v) sodium carbonate in 0.1 N NaOH in 1:50 (v/v) ratio.

After incubation for 10 minutes at room temperature, 0.5 ml of 1 N Folin's reagent was added. The contents were rapidly mixed and colour intensity read after 30 minutes against a reagent blank at 660 nm. A standard curve prepared using BSA was used to calculate the concentration of protein.

Sulfhydryl Estimation

Free sulfhydryl in native and modified papain was estimated according to the method of Ellman (1959) using standard plot of glutathione (3 mg/100 ml of 100 mM Tris buffer pH 8). To the appropriate amount of papain was added 0.1 M DTNB in 0.1 M Tris-EDTA buffer pH 8.0 in a total volume of 3.1 ml and the absorbance measured after 15 min. The appearance of thionitrobenzoate ion was monitored at 410 nm in DU 40 spectrophotometer.

ENZYME ASSAY

Casein Hydrolysis Activity

The proteolytic activity of papain was determined by using Casein as substrate according to the method of Kunitz (1947) with slight modification. The enzyme was taken in 10 mM phosphate buffer pH 8.6 and activated for 10 minutes at 37°C with 0.2 ml of 0.05 M cysteine and 0.02 M EDTA after adjusting their pH to 8.6. The volume of the reaction mixture was made up to 1.0 ml. 1.0 ml of 2% (w/v) casein (pH adjusted to 8.6) was incubated with the activated enzyme at 37°C for 15 minutes after 30 seconds interval. Reaction

was terminated with 1.0 ml of 10% (w/v) TCA. After 30 minutes standing at room temperature the tubes were centrifuged at 3000 rpm for 10 minutes. Colour of aliquots of supernatant were developed by Lowry's procedure (1951). Control was prepared in an identical manner except that substrate casein was added after adding trichloroacetic acid and the blue colour was read at 660 nm.

One unit of enzyme activity is the amount of enzyme which under experimental conditions give rise to the digestion product per minute at 37°C.

Amidase Activity (BAPNA hydrolysis activity)

The amidase activity of native, insolubilized and immobilized papain was determined by the method of Erlanger *et al* (1961). The method is based upon the cleavage of an amide bond in a synthetic substrate BAPNA. To 0.250 ml papain increasing volume (0.150 ml - 0.950 ml) of BAPNA was added in each tube. Final volume was adjusted to 1.5 ml with distilled water. The tubes were incubated at 37°C for 30 minutes. Reaction was terminated after 30 minutes by the addition of 0.5 ml of 30% acetic acid. The quantity of liberated p-nitroaniline was estimated spectrophotometrically at 410 nm.

One unit of BAPNA activity is the amount which will hydrolyse 1 micromole of substrate per minute under above conditions.

RESULTS

I. MODIFICATION OF PAPAIN

The commercially available papain was first dialyzed against 10 mM phosphate buffer pH 8.6 in order to eliminate the salt and other impurities. Free sulfhydryl groups present on the active site of papain were modified by the treatment of iodoacetic acid and iodoacetamide using the procedure of Crestfield et al. (1963). The number of sulfhydryl groups present on the active site of native and modified papain were calculated from the standard plot of glutathione (Fig. 1). The values shown in Table 1 clearly indicate SH group modification. The electrophoretic pattern of the papain modified with iodoacetic acid and iodoacetamide remained unaltered and was comparable with the native papain (Fig. 2).

Gel Filtration Pattern of Native and Modified Papain

Gel filtration pattern of native, modified papain and marker proteins is shown in Fig. 3. Sephadex G-100 column equilibrated with 10 mM phosphate buffer pH 8.6 was used to calibrate with four marker proteins, cytochrome C, ribonuclease A, α-chymotrypsinogen A and BSA.10 mg of each protein was applied and eluted with the same buffer at a flow rate of 24 ml/hr and 3 ml fractions were collected. Protein was determined by the procedure of Lowry et al (191). Native and modified papain were eluted with the same buffer, and activity as well as protein concentration were determined. Blue dextran was

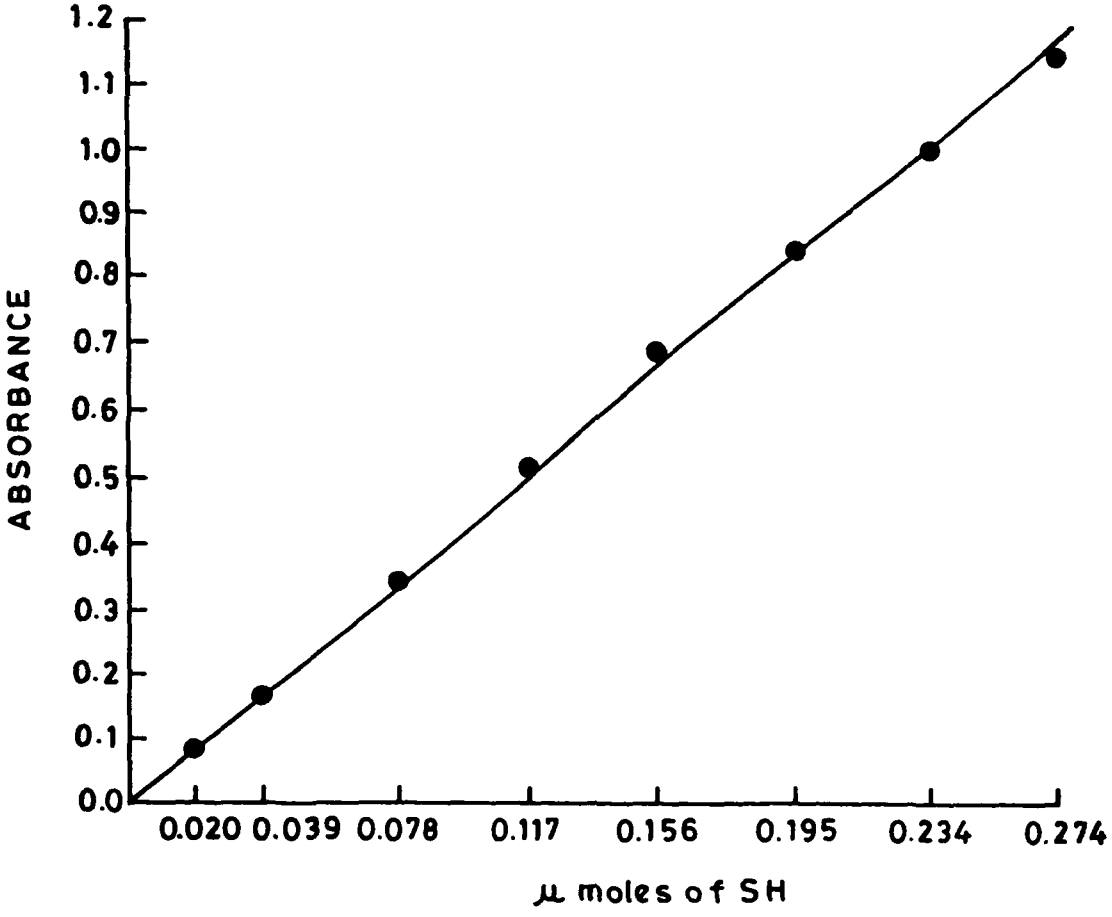

ABSORBANCE
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.020
0.039
0.078
0.117
0.156
0.195
0.234
0.274
μ moles of SH

Table I. **Sulfhydryl groups in native and modified papain.**

Aliquots of native and modified papain (1560 μg) were analyzed for SH groups using DTNB as described in the text. Number of μ mole of SH groups/μ mole of protein were determined from the glutathione standard plot.

Papain Preparations	μ mole of SH/μ mole of Protein
Native papain	1
Iodoacetamide treated papain	0
Iodoacetic acid treated papain	0

Fig. 2 Polyacrylamide gel electrophoresis of native and modified papain.

Native and modified papain (300 μg) preparations were subjected to electrophoresis in 10% polyacrylamide gel as described in the text. The gels were stained with the silver nitrate method of Merril et al (1982).

a - native papain

b - iodoacetic acid treated papain

c - native papain

d - iodoacetamide treated papain

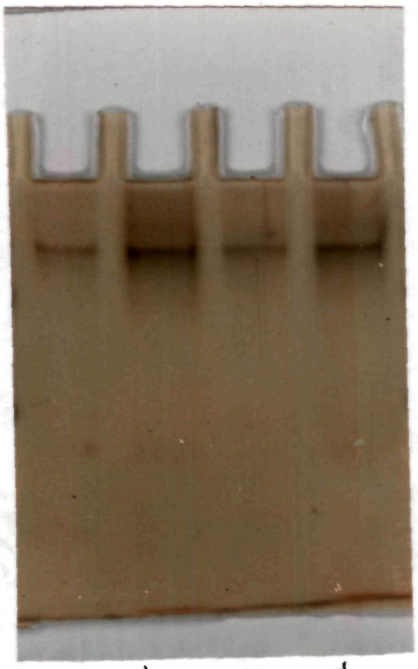

a
b
c
d

Fig. 3 Gel filtration behaviour of Marker proteins and papain preparations.

About 10 mg of each protein was chromatographed on the column (2 cm x 62 cm) and eluted with 10 mM phosphate buffer pH 8.6. 3 ml fractions were collected at a flow rate of 24 ml/hr.

A - cytochrome C

B - Ribonuclease A

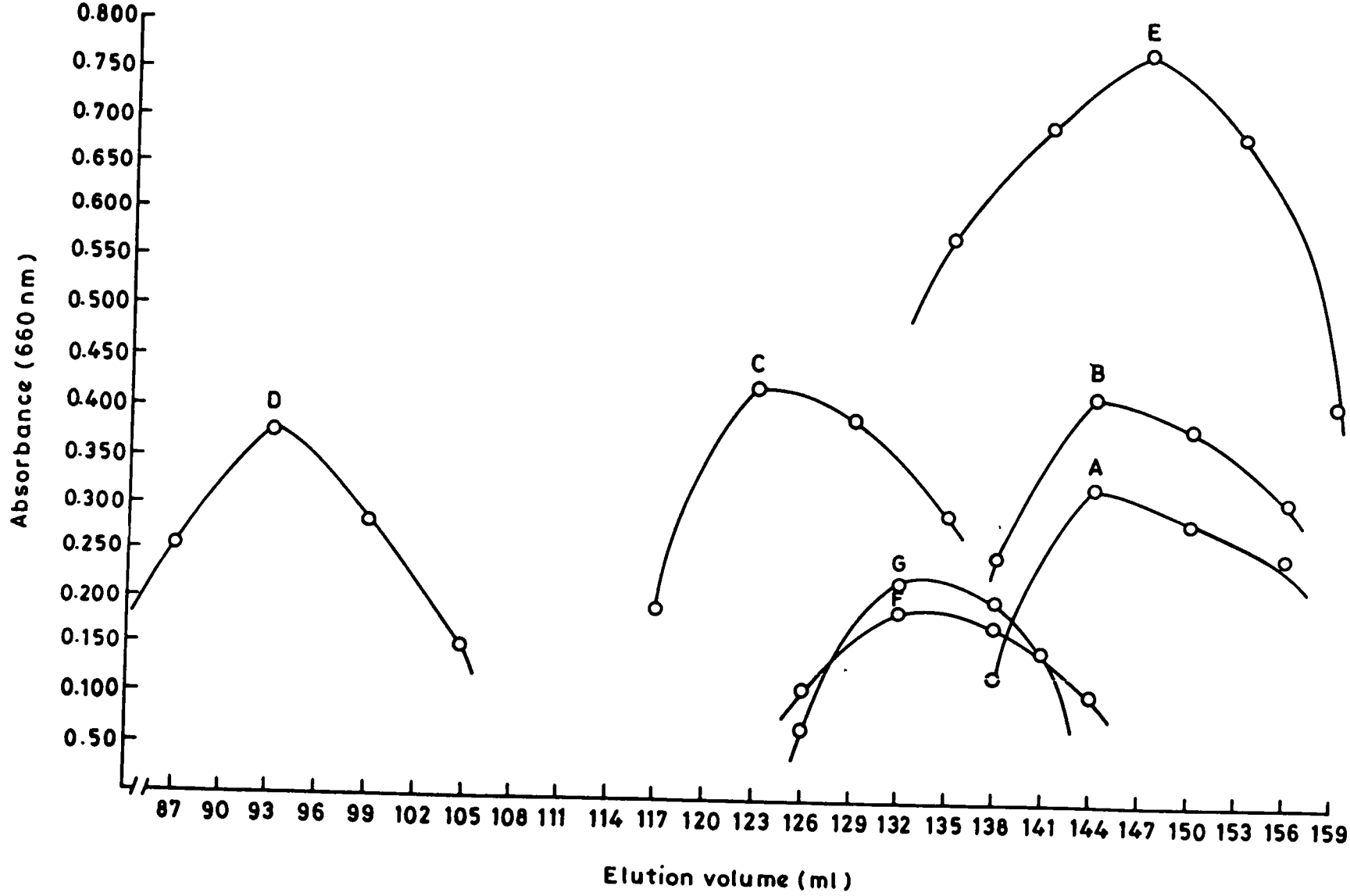
C - α-Chymotrypsinogen A

D - BSA

E - Native Papain

F - Iodoacetamide treated papain

G - Iodoacetic acid treated papain


Absorbance (660 nm)
0.800
0.750
0.700
0.650
0.600
0.550
0.500
0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.50
87 90 93 96 99 102 105 108 111 114 117 120 123 126 129 132 135 138 141 144 147 150 153 156 159
Elution volume (ml)
A
B
C
D
E
F
G

used to determine the void volume (V_0), inner volume (V_i) was calculated by the formula V_i = awr. The total volume V_t was determined directly with water.

The same elution profile was used to determine the Stoke's radius of native, iodoacetamide and iodoacitic acid treated papain. The data was processed by the following expression -

$$K_d = \frac{V_e - V_o}{V_i} \quad \text{.....(1)}$$

$$K_{av} = \frac{V_e - V_o}{V_i - V_o} \quad \text{.....(2)}$$

Where K_d is the distribution coefficient and K_{av} is the available distribution coefficient. With the help of equation 1 and 2, K_d and K_{av} of marker proteins and papain preparations was calculated and shown in Table II. The data was plotted according to Laurent and Killander (1964) to determine the Stoke's radius (Fig. 4).

II. IMMUNIZATION STUDIES

(i) Immunodiffusion and ELISA Technique for the Detection of Antibodies : The antisera raised against native, iodoacetamide and iodoacetic acid treated papain produced precipitating antibodies as evident from the immunodiffusion studies. A single precipitin line was observed on immunodiffusion of native and modified papain against their specific antisera

Table II. Various parameters for marker proteins and papain preparations calculated from Gel filtration experiment.

The Kd, Kav and Stoke's radius of different papain preparations were calculated accordingly as described in the text.

	Kd	Kav	(-log Kav)^{½}	Stoke's radius (nm)	Ve/Vo
1. Cytochrome C	0.600	0.597	0.473	2.705	2.087
2. Ribonuclease A	0.600	0.597	0.473	2.705	2.087
3. α-Chymotrypsinogen A	0.432	0.430	0.603	3.397	1.783
4. BSA	0.192	0.191	0.846	4.685	1.341
5. Native papain	0.624	0.621	0.454	2.652	2.130
6. Iodoacetamide treated papain	0.504	0.502	0.547	3.101	1.913
7. Iodoacetic acid treated papain	0.504	0.502	0.547	3.101	1.913

Fig. 4 Treatment of Gel filtration data of proteins according to Laurent and Killander.

The gel filtration data of Table-II was treated according to the correlation of Laurent and Killander (1964). Plot of $(-\log K_{av})^{\frac{1}{2}}$ vs Stoke's radius gave the straight line by the least square method.

(i) Cytochrome C

(ii) Ribonuclease A

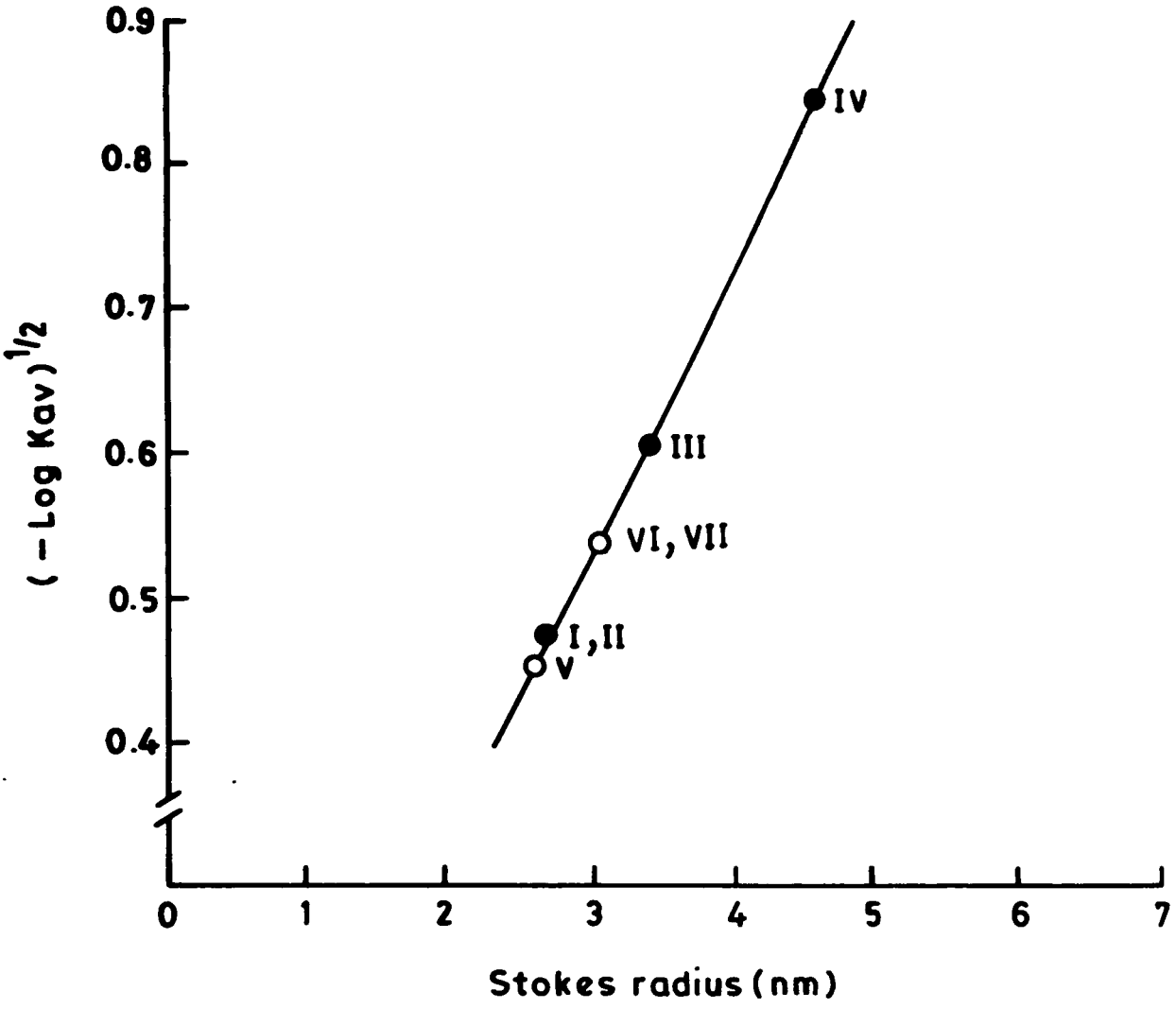
(iii) α-Chymotrypsinogen A

(iv) BSA

(v) Native papain

(vi) Iodoacetamide treated papain

(vii) Iodoacetic acid treated papain


0.9
0.8
0.7
0.6
0.5
0.4
(-Log Kav)^1/2
IV
III
VI, VII
I, II
V
0
1
2
3
4
5
6
7
Stokes radius (nm)

after overnight incubation (Fig. 5, 6 & 7). The antisera of iodoacetic acid and iodoacetamide treated papain also cross reacted with the native papain antigen (Fig. 8).

The ELISA technique was used to check the titre of antisera produced against their specific antigens. As shown in Fig. 9, 10 and 11 very high titre of antibodies raised against native, iodoacetamide and iodoacetic acid modified papain was observed.

(ii) Purification of IgG : In order to eliminate the serum contaminants, IgG fraction was obtained by ammonium sulphate precipitation (0-40% cut). To further purify the IgG, DEAE cellulose column was used and purified IgG was obtained. The electrophoretic pattern of antiserum, supernatant from ammonium sulphate cut, ammonium sulphate precipitated IgG fraction and DEAE cellulose purified IgG is shown in Fig. 12. A clear IgG band was visualized in lane 3 and 4, while IgG band was missing from the lane 2, suggesting the precipitation of IgG at the reported concentration of ammonium sulphate.

The crossreactivity of antiserum, supernatant and IgG fraction from ammonium sulphate precipitation against native papain is shown in Fig. 13. A single precipitin line was obtained in the wells containing antiserum and IgG fractions, while no precipitin line was observed in the wells loaded with supernatant.

Fig. 5 Ouchterlony immuno-double diffusion test of antiserum from native papain treated rabbits.

Cross reactivity of native papain with its antiserum was performed in 0.1% agarose gel as described in the text. The central well contained native papain (Ag) while peripheral wells contained.

(1) Undiluted antiserum

(2) 1:2 diluted antiserum

(3) 1:4 diluted antiserum

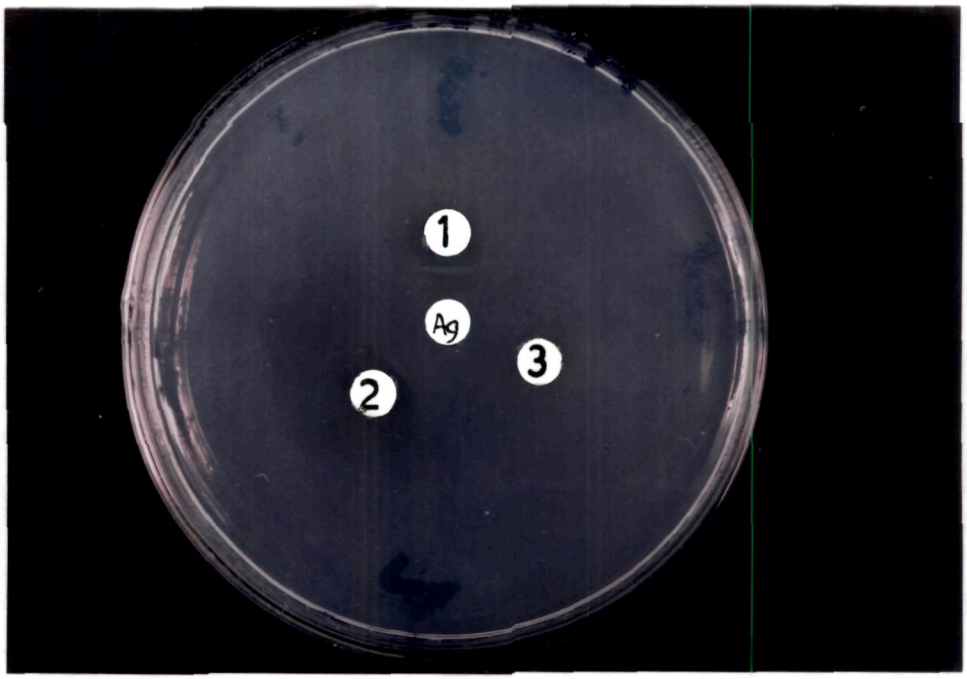

1
Ag
2
3

Fig. 6 Ouchterlony immuno-double diffusion test of antiserum from iodoacetamide modified papain treated rabbits.

Cross reactivity of iodoacetamide treated papain with its antiserum was performed in 0.1% agarose gel as described in the text. The central well contained treated papain (Ag) while peripheral wells contained.

(1) Undiluted antiserum

(2) 1:2 diluted antiserum

(3) 1:4 diluted antiserum

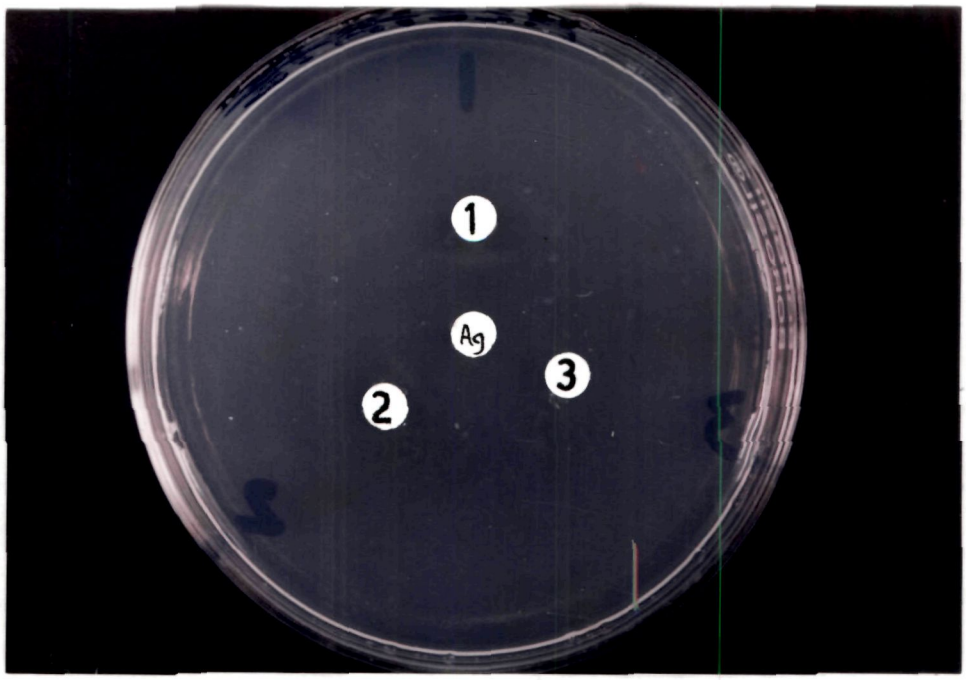

1
Ag
2
3

Fig. 7 Ouchterlony immuno-double diffusion test of antiserum from iodoacetic acid modified papain treated rabbits.

Cross reactivity of iodoacetic acid treated papain with its antiserum was performed in 0.1% agarose gel as described in the text. The central well contained treated papain (Ag) while peripheral wells contained.

(1) Undiluted antiserum

(2) 1:2 diluted antiserum

(3) 1:4 diluted antiserum

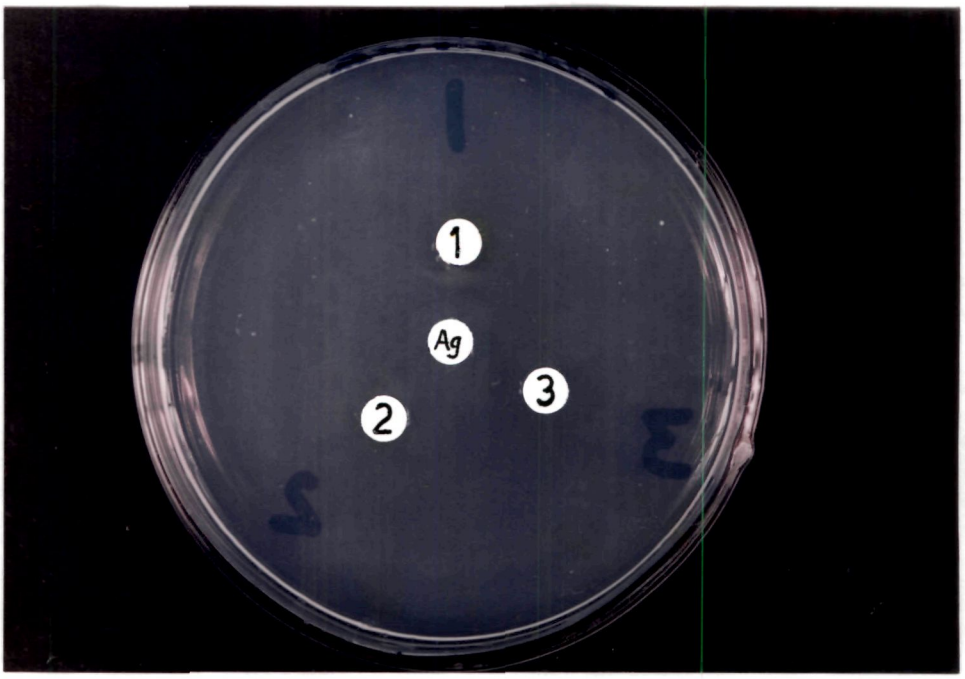

1
Ag
2
3

Fig. 8 Ouchterlony immuno-double diffusion test of antisera from native, and modified papain treated rabbits.

Cross reactivity of native papain with the antisera of native and modified papain was performed in 0.1% agarose gel as described in the text. The central well contained native papain (Ag), while peripheral wells contained.

(1) antiserum against native papain

(2) antiserum against iodoacetamide treated papain

(3) antiserum against iodoacetic acid treated papain

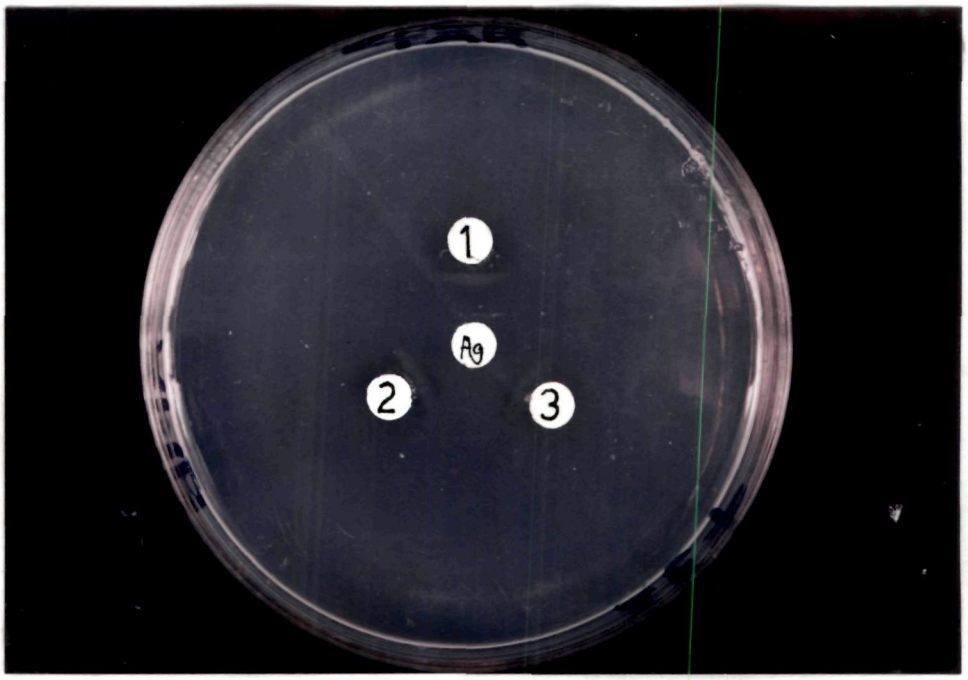

1
Ag
2
3

Fig. 9 Direct binding ELISA of native papain antiserum.

Pre-immunized and native papain immunized rabbit antisera were tested by direct binding ELISA at different dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800). The wells were coated with papain containing 50 μg protein/ml in 10 mM phosphate buffer pH 8.6. The negative logarithm of serum dilution versus the absorbance at 410 nm are plotted.

(● - ●) Immunized serum

(o - o) Pre-immunized serum

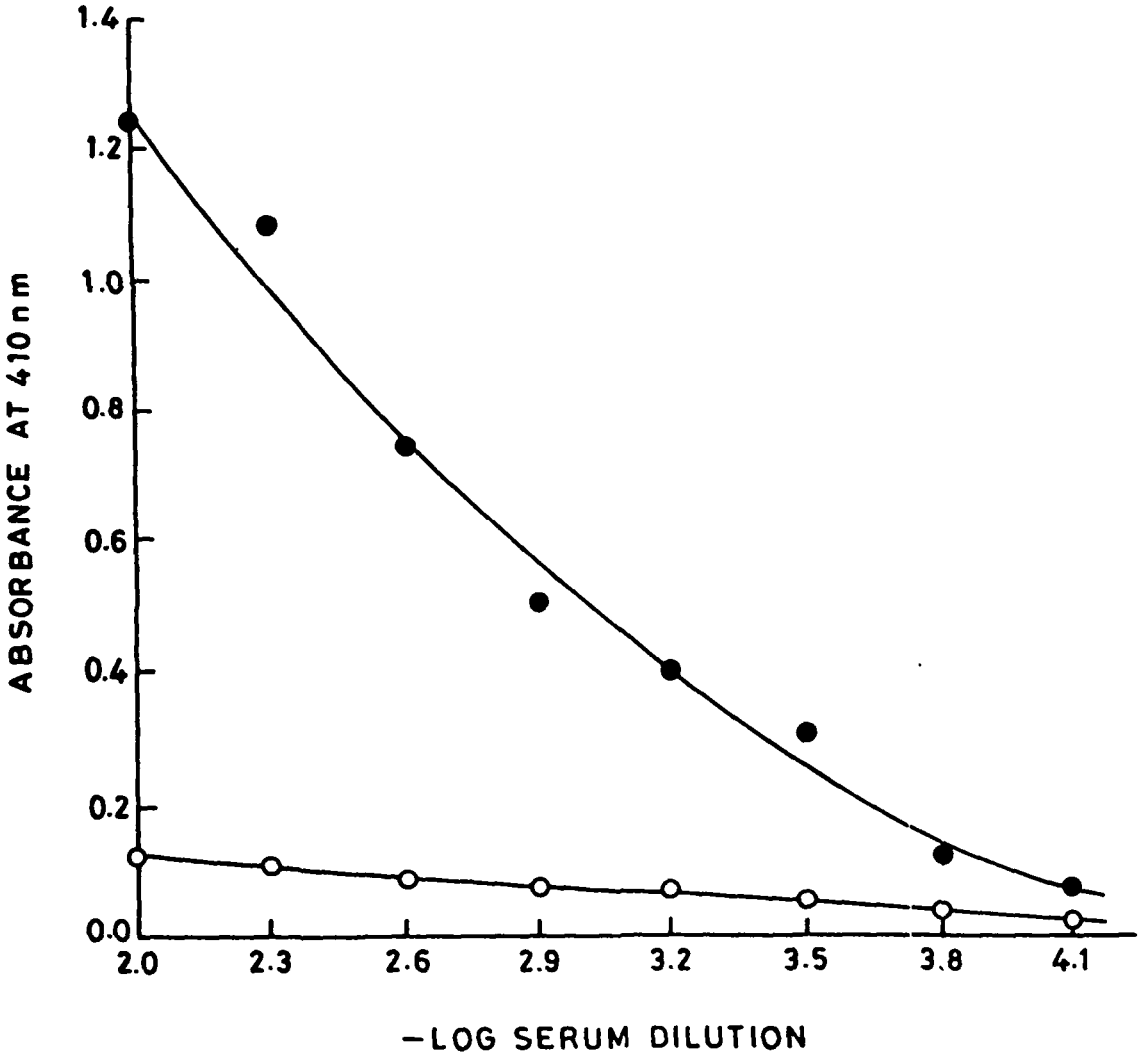

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE AT 410 nm
2.0
2.3
2.6
2.9
3.2
3.5
3.8
4.1
−LOG SERUM DILUTION

Fig. 10 Direct binding ELISA of iodoacetamide treated papain antiserum.

Pre-immunized and (iodoacetamide treated) papain immunized rabbit antisera were tested by direct binding ELISA at different dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800). The wells were coated with papain containing 50 μg protein/ml in 10 mM phosphate buffer pH 8.6. The negative logarithm of serum dilution versus the absorbance at 410 nm are plotted.

(● - ●) Immunized serum

(o - o) Pre-immunized serum

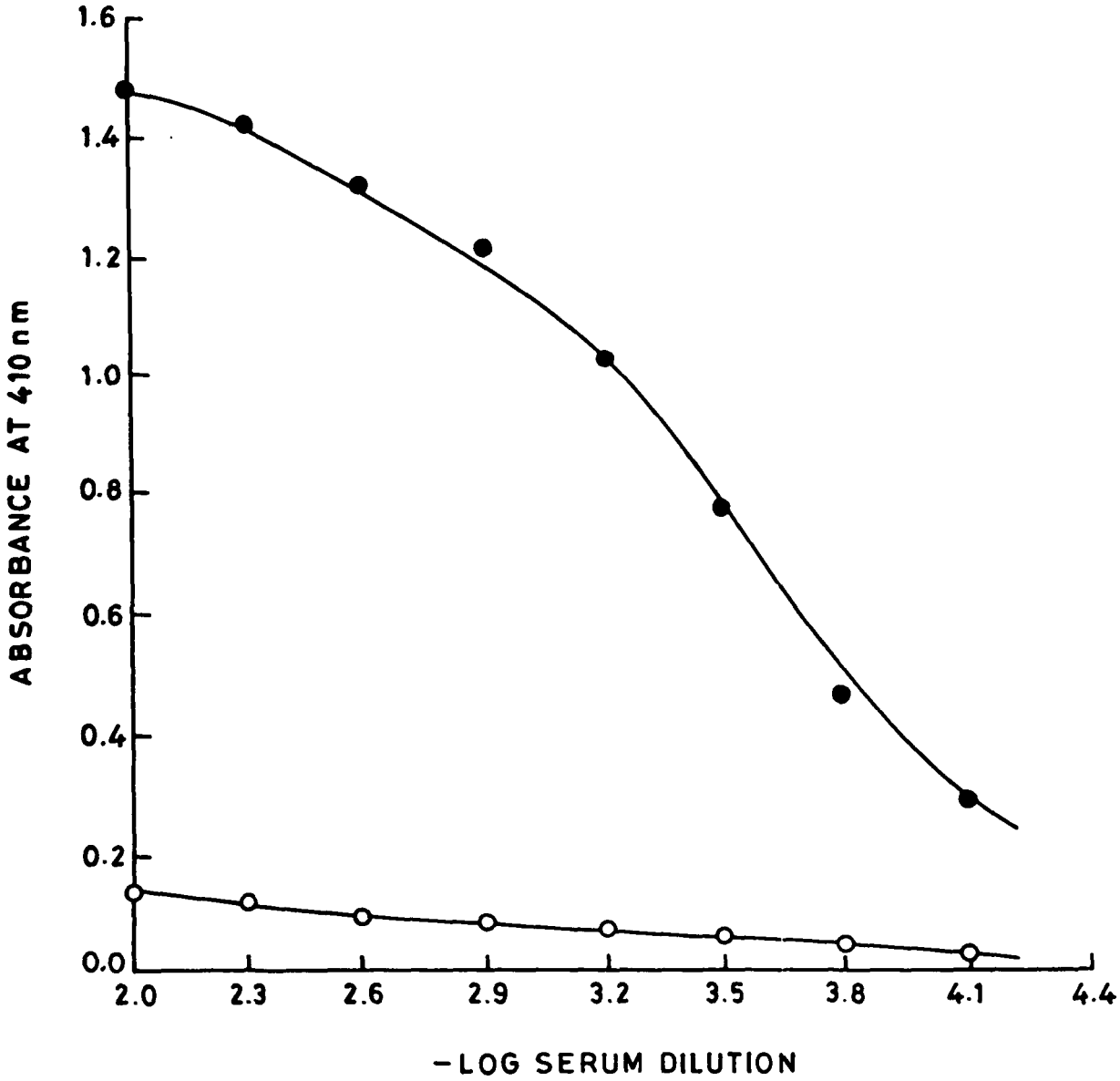

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE AT 410 nm
2.0
2.3
2.6
2.9
3.2
3.5
3.8
4.1
4.4
-LOG SERUM DILUTION

Fig. 11 Direct binding ELISA of iodoacetic acid treated papain antiserum.

Pre-immunized and (iodoacetic acid treated) papain immunized rabbit antisera were tested by direct binding ELISA at different dilutions (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800). The wells were coated with papain containing 50 μg protein/ml in 10 mM phosphate buffer pH 8.6. The negative logarithm of serum dilution versus the absorbance at 410 nm are plotted.

(● - ●) Immunized serum

(o - o) Preimmunized serum

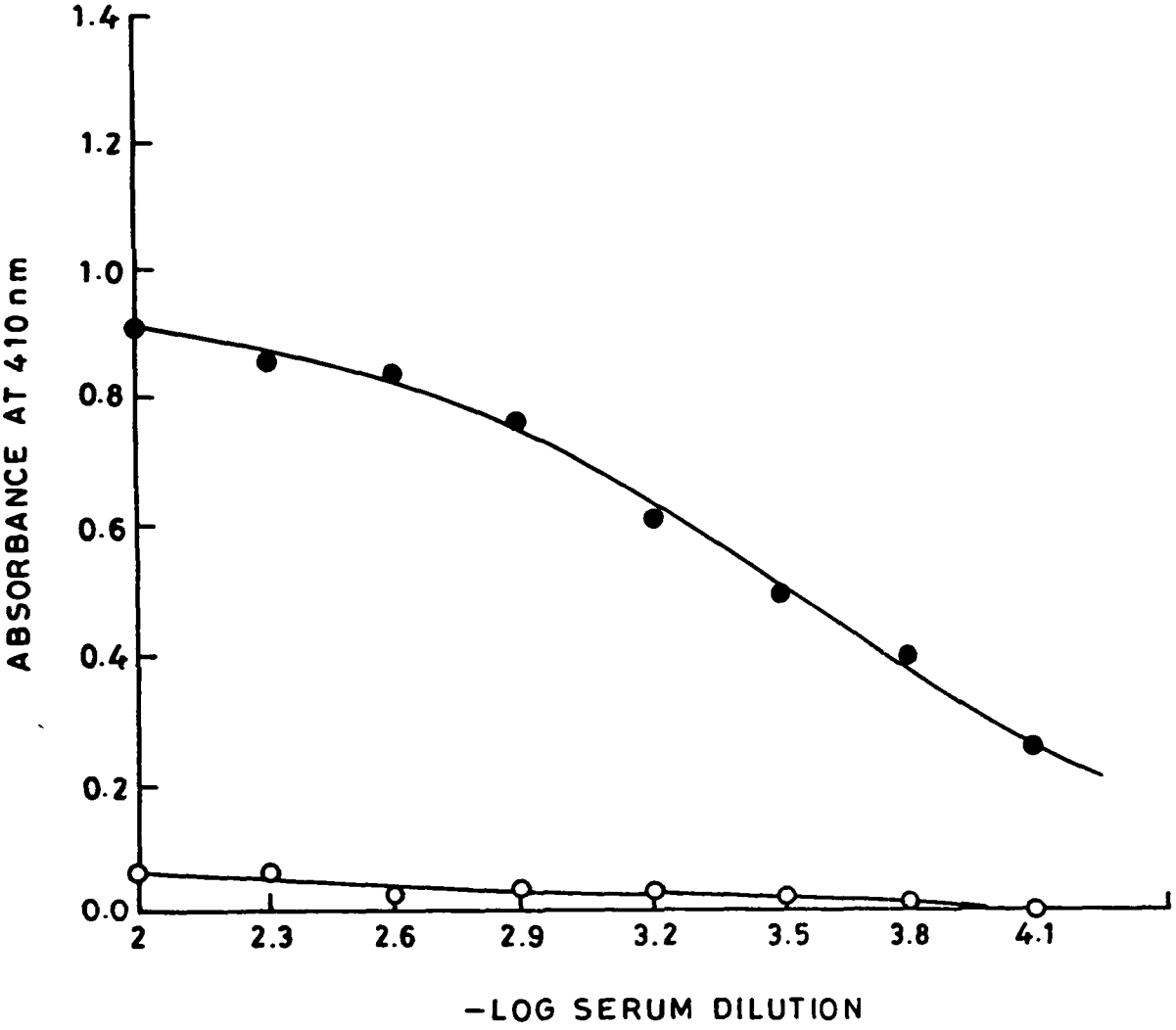

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ABSORBANCE AT 410 nm
2
2.3
2.6
2.9
3.2
3.5
3.8
4.1
-LOG SERUM DILUTION

Fig. 12 Polyacrylamide gel electrophoresis to show a simple IgG fractionation scheme.

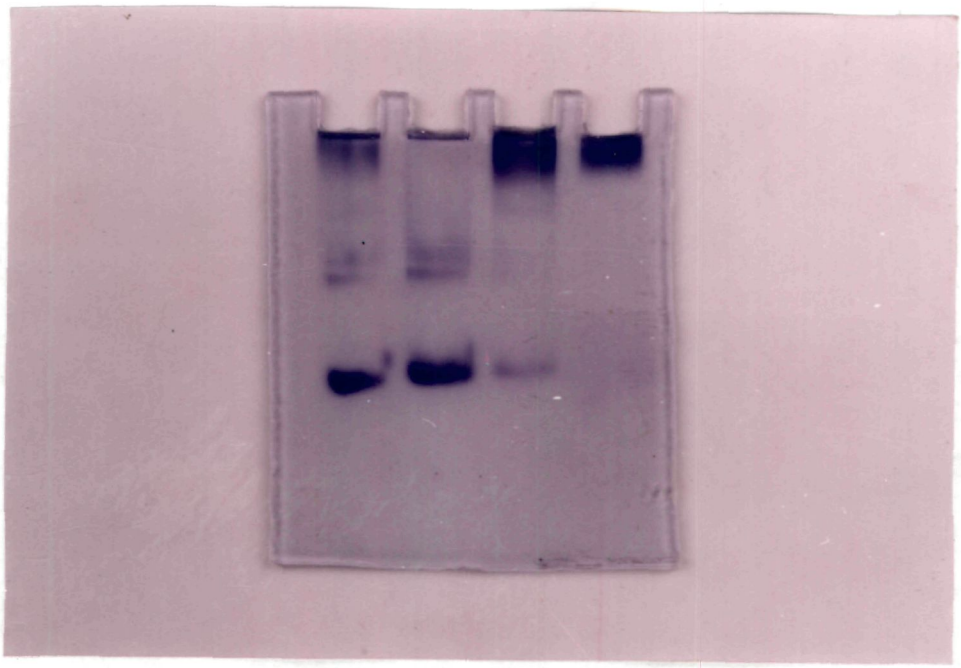
The different aliquots of antipapain antiserum, supernatant of ammonium sulphate precipitate, ammonium sulphate precipitated IgG and purified IgG on DEAE column were electrophoresed in 7.5% polyacrylamide gel as described in the text. The staining was done with coomasie brilliant blue.

Lane 1 - Antipapain antiserum

Lane 2 - Supernatant from ammonium sulphate precipitated fraction

Lane 3 - Ammonium sulphate precipitated IgG

Lane 4 - DEAE purified IgG



1 2 3 4

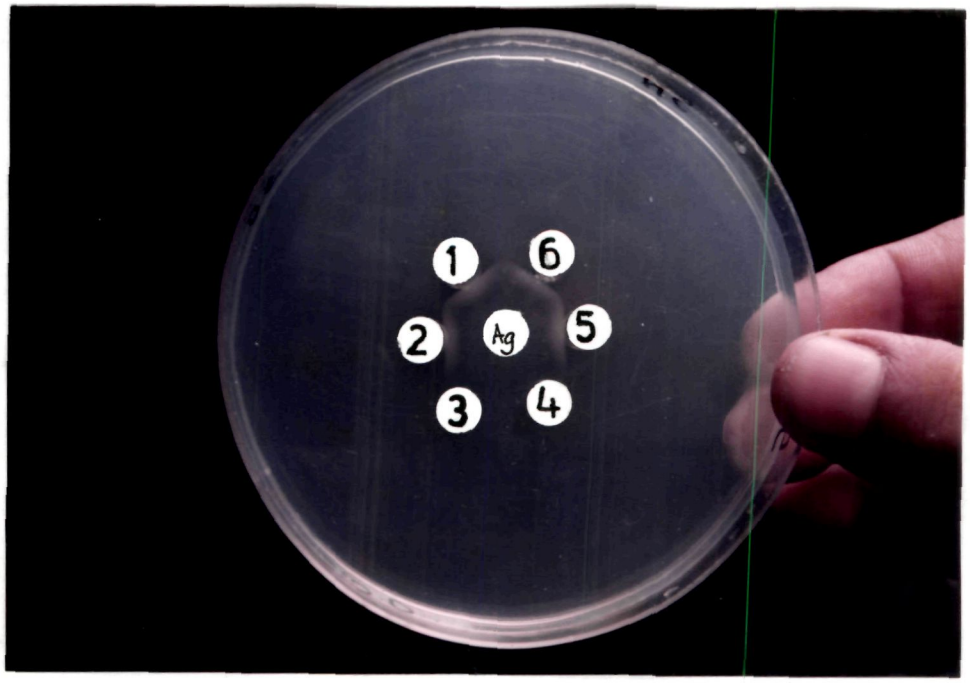
Fig. 13 Precipitin reaction of native papain antiserum, supernatant of ammonium sulphate precipitate and ammonium sulphate precipitated IgG.

Precipitin reaction was performed in 0.1% agarose containing 0.9% NaCl. Central well contained native papain (Ag). Peripheral wells contained.

(1, 2) Antiserum

(3, 4) Supernatant

(5, 6) IgG


1
6
2
Ag
5
3
4

III. INSOLUBILIZATION OF PAPAIN WITH ANTIPAPAIN ANTISERUM / IgG FRACTION

Considering the non-inhibitory nature, native papain antiserum was selected for the insolubilization studies. Table III shows the immunoprecipitation of papain at two different concentrations with the fixed amount of antipapain antiserum and preparations obtained designated as (A) and (B). Most of the papain activity was retained by these complexes as evident from the high η values (0.75 and 0.96 for preparations (A) and (B) respectively). The η value of papain in immunocomplex (A) was lower than those of (B). Similar set of experiment was repeated with IgG fraction isolated from the antipapain antiserum by precipitating with ammonium sulfate in order to avoid any effect of serum contaminants during the preparation. The results are shown in Table IV with slightly improved η values of the two papain preparations designated as (A) and (B) (η values were 0.85 and 0.98 for preparations (A) and (B) respectively). The antibody to enzyme ratio in preparation (A) was lower while it was higher in the preparation (B).

IV. STUDIES ON PAPAIN-ANTIBODY ADDUCTS

(i) Effect of Temperature : The thermostability of two papain preparations (A) and (B) insolubilized with antipapain antiserum and isolated IgG was investigated at 75°C for different time intervals. The results of preparation (A)

Table III. **Immunoprecipitation of papain with antipapain antiserum.**

Each value represents the average result of atleast three experiments performed in duplicate. In order to obtain 'theoretical' binding, units unbound and those in the washing were substracted from the added units. An appropriate aliquot of the insolubilized preparation was assayed to obtain the 'Actual' activity. η values represent the ratio of actual and theoretical activity of papain insolubilized with antipapain antiserum.

	Units added	Units in washes	Insolubilized Activity (Units)		η value
			Theoretical (a)	Actual (b)	b/a
*Preparation (A)	59640	43640	16000	12000	0.75
*Preparation (B)	15840	12169	3671	3520	0.96

*Described in the text.

Table IV. Immunoprecipitation of papain with IgG isolated from immune sera.

Each value represents the average result of atleast three experiments performed in duplicate. In order to obtain 'theoretical' binding, units unbound and those in the washing were substracted from the added units. An appropriate aliquot of the insolubilized preparation was assayed to obtain the 'actual' activity. η values represent the ratio of 'actual' and 'theoretical' activity of papain insolubilized with IgG.

	Units added	Units in washes	Insolubilized Activity (Units)		η value
			Theoretical (a)	Actual (b)	b/a
*Preparation (A)	49600	36240	13360	11400	0.85
*Preparation (B)	23260	17040	6220	6064	0.98

*Described in the text.

complexed with antipapain antiserum and IgG are shown in Fig. 14 (a and b) respectively. As it is clearly evident from the figure the immunoprecipitate (box a) exhibited marked increase in thermostability and retained 75% activity after two hours incubation while its soluble counterpart retained only 25% activity. The results of immunoprecipitate complexed with IgG are shown in the box (b) with slightly improved thermostability than (a) for the same duration. The results of thermostability of immunoprecipitate using preparation (B) are shown in Fig. 15 (a and b). The immunoprecipitate complexed with antiserum as well as IgG were most stable retaining almost 100% activity after 2 hours incubation at 75°C, and its soluble counterpart retained 80% activity for the same duration.

Fig. 16 and 17 (a & b) show the thermostability curves of papain at different temperatures for 30 minutes in its native and complexed form. Both immunocomplexes of preparations (A) and (B) with antiserum as well as IgG were resistant to denaturation even at 90°C, though the immunocomplex of preparation (B) retained 85 and 90% activity at 90°C, when incubated for 30 minutes. The native papain used alongwith the preparation (B) also retained 70% activity for the same duration, while only 40% activity could be recovered in the soluble counterpart of preparation (A).

(ii) Effect of Urea : The effect of urea on native, insolubilized preparations (A) and (B) having low and high antibody to enzyme ratios respectively was

Fig. 14 Thermal inactivation of soluble and insolubilized papain preparations (A).

Thermal stability of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), was determined by incubating these preparations for various durations at 75°C. Papain activity was determined at the end of the treatment under standard conditions.

(o) soluble papain,

(●) insolubilized papain.

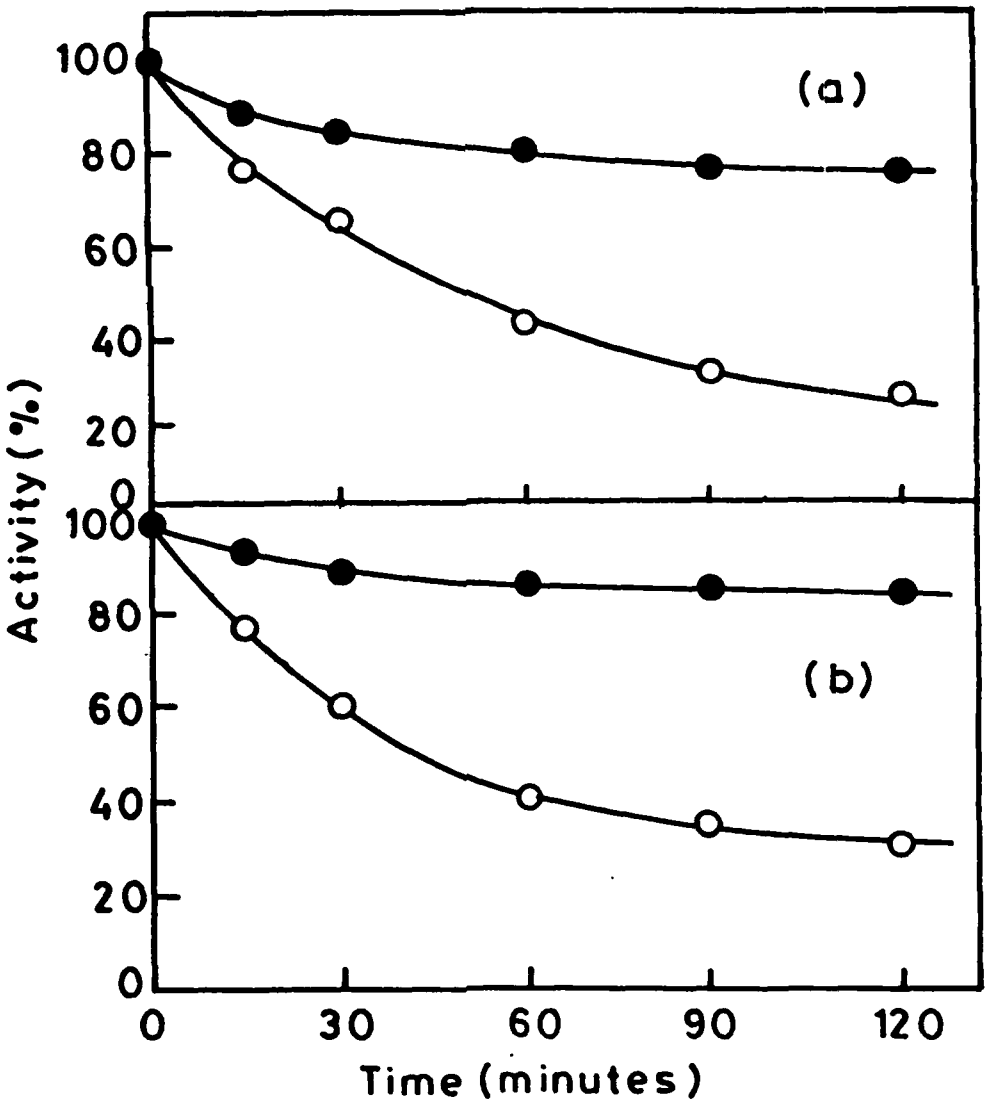

100
80
60
40
20
0
(a)
100
80
60
40
20
0
(b)
Activity (%)
0
30
60
90
120
Time (minutes)

Fig. 15 Thermal inactivation of soluble and insolubilized papain preparations (B).

Thermal stability of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), was determined by incubating these preparations for various durations at 75°C. Papain activity was determined at the end of the treatment under standard conditions.

(Δ) soluble papain,

(▲) insolubilized papain.

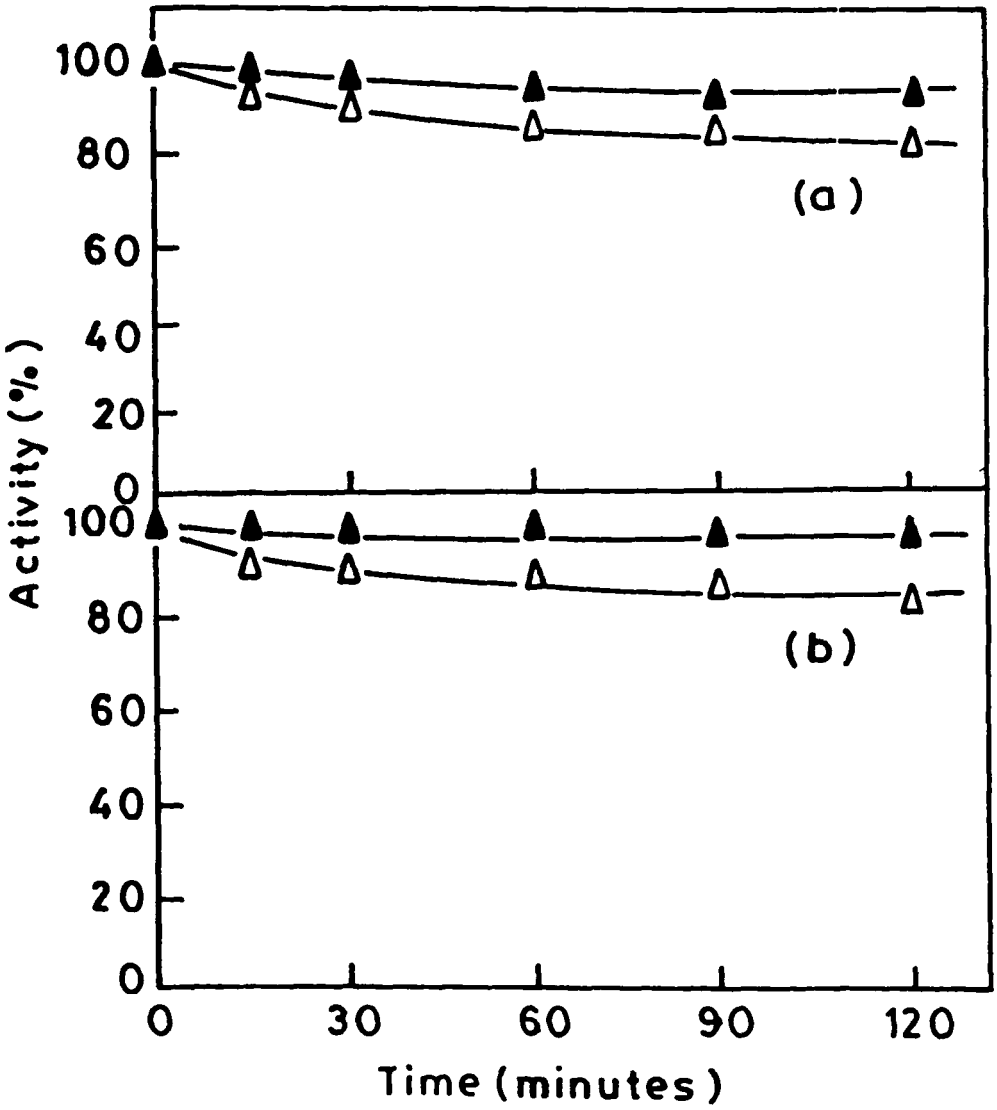

100
80
60
40
20
0
(a)
100
80
60
40
20
0
(b)
Activity (%)
0
30
60
90
120
Time (minutes)

Fig. 16 Effect of pretreatment at various temperatures on soluble and insolubilized papain preparations (A).

Thermal inactivation of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), was determined by incubating these preparations at various temperatures for 30 minutes, cooled and activity was determined under standard conditions.

(o) soluble papain,

(●) insolubilized papain.

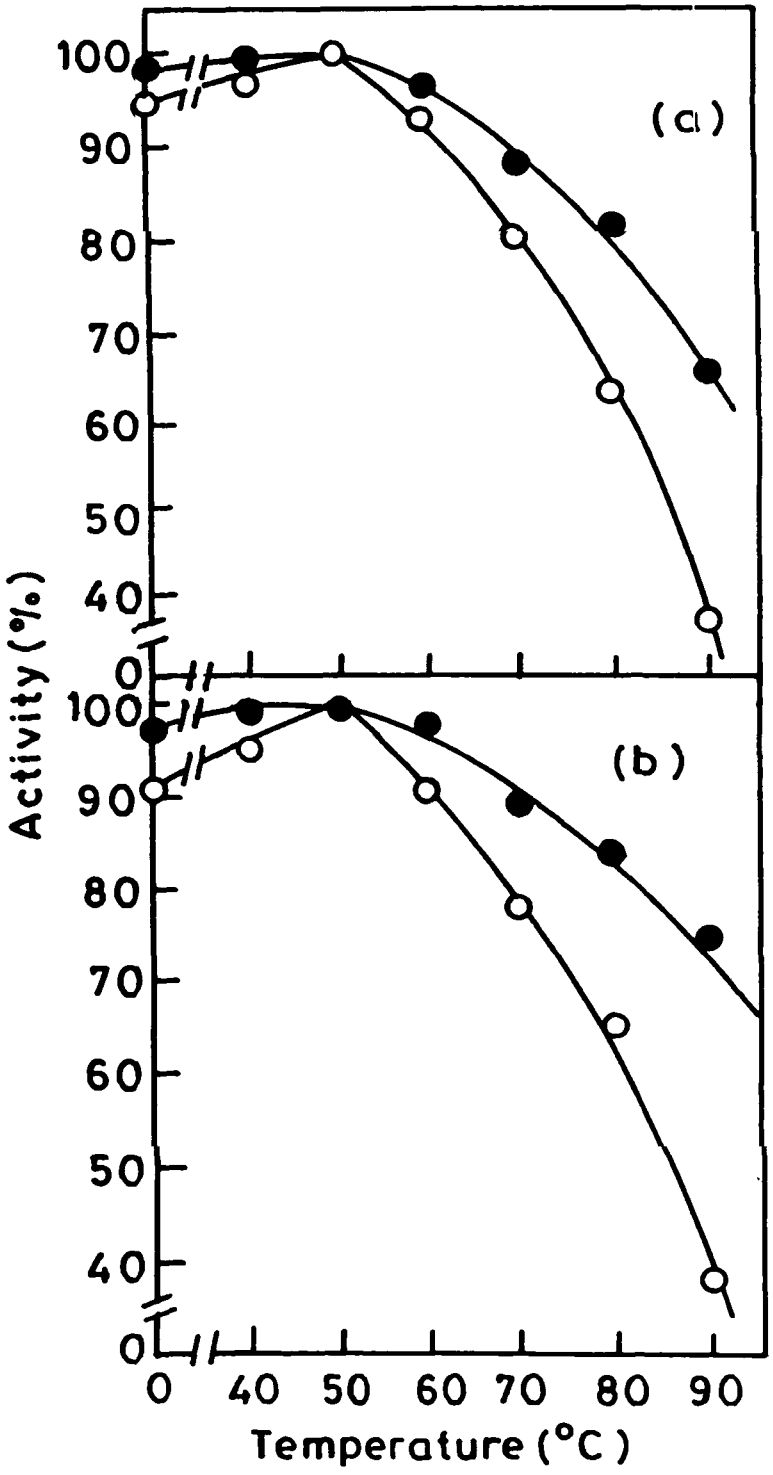
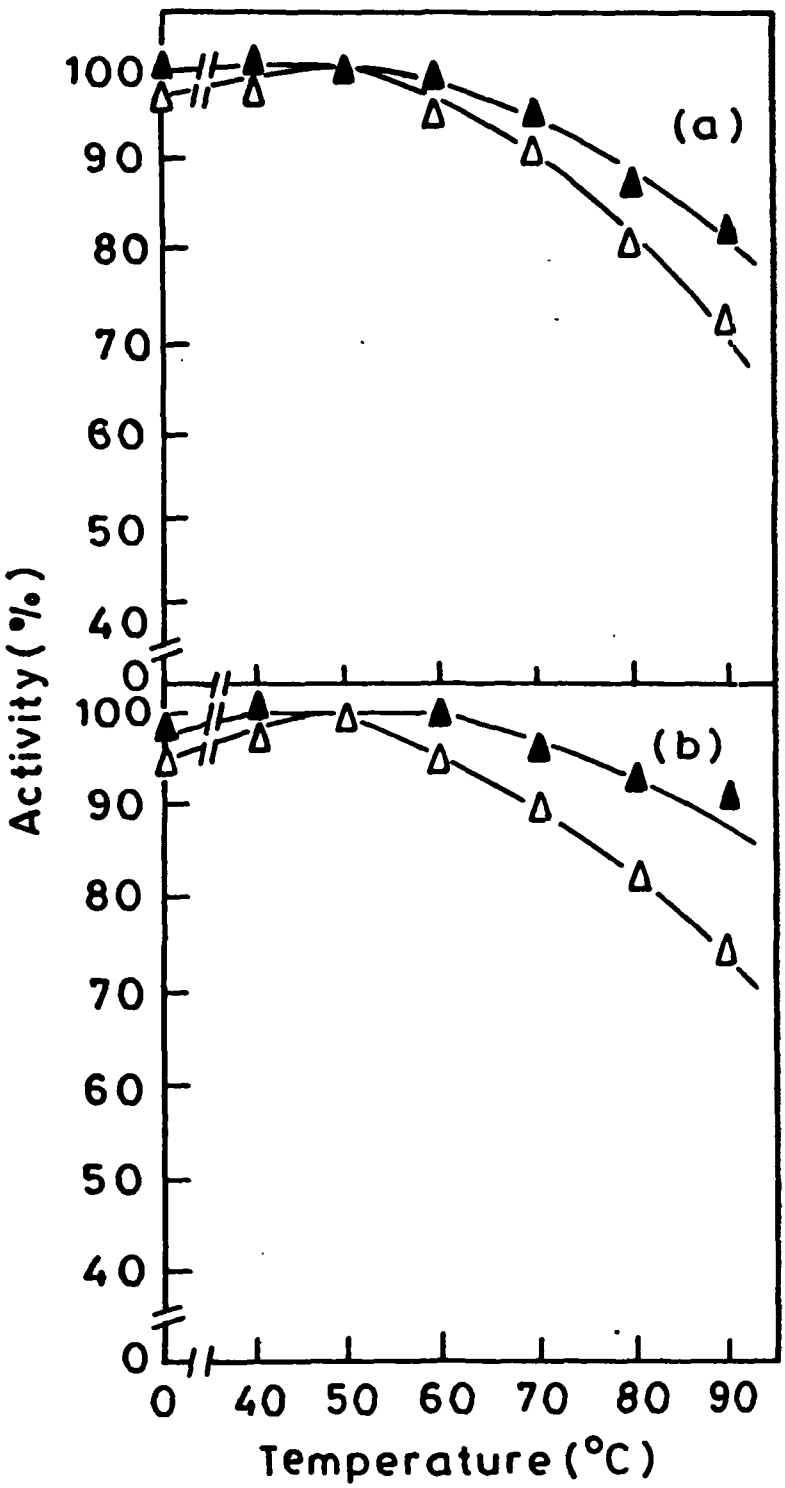

(a)
(b)
100
90
80
70
60
50
40
0
Activity (%)
0 40 50 60 70 80 90
Temperature (°C)

Fig. 17 Effect of pretreatment at various temperatures on soluble and insolubilized papain preparations (B).

Thermal inactivation of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), was determined by incubating these preparations at various temperatures for 30 minutes, cooled and activity was determined under standard conditions.

(Δ) soluble papain,

(▲) insolubilized papain.


100
90
80
70
60
50
40
0
(a)
100
90
80
70
60
50
40
0
(b)
Activity (%)
0
40
50
60
70
80
90
Temperature (°C)

also determined. As evident from Fig. 18 (a and b), the native papain, a counterpart of preparation (A), retained 60% activity after incubating with 4 M urea for 150 minutes. However, significant activities of insolubilized preparations persisted over this period. The retained activity was higher in the immunocomplex where antiserum was replaced with IgG, maintaining same (low) antibody to enzyme ratio. The results of preparation (B) with high antibody to enzyme ratio and its soluble counterpart are shown in Fig. 19 (a and b). Both immunocomplexes of preparation (B) obtained by using antiserum and IgG retained more activity in comparison to the immunocomplexes of preparation (A). At the same time the soluble counterpart of preparations (B) was also more resistant to urea denaturation in comparison to the native papain used with both the preparations of complex (A). The 82% activity was retained by the soluble papain of preparation (B), while incubating with 4 M urea for 150 minutes.

(iii) Effect of pH : There was no significant alteration in the pH activity profile of papain as a result of immunoprecipitation (Figs. 20 and 21 (a & b)). There was neither any shift in the pH optima nor any significant broadening of the pH activity curve in both the immunocomplexes as well as their soluble counterparts. The replacement of the antiserum with IgG during the formation of immunocomplexes did not produce any alteration in the pH activity curve.

Fig. 18 Denaturant stability of soluble and insolubilized papain preparations (A).

Stability of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), towards denaturants was determined by incubating these preparations in 4 M urea at 37°C for the indicated durations, and activity was determined under standard conditions.

(o) soluble papain,

(●) insolubilized papain.

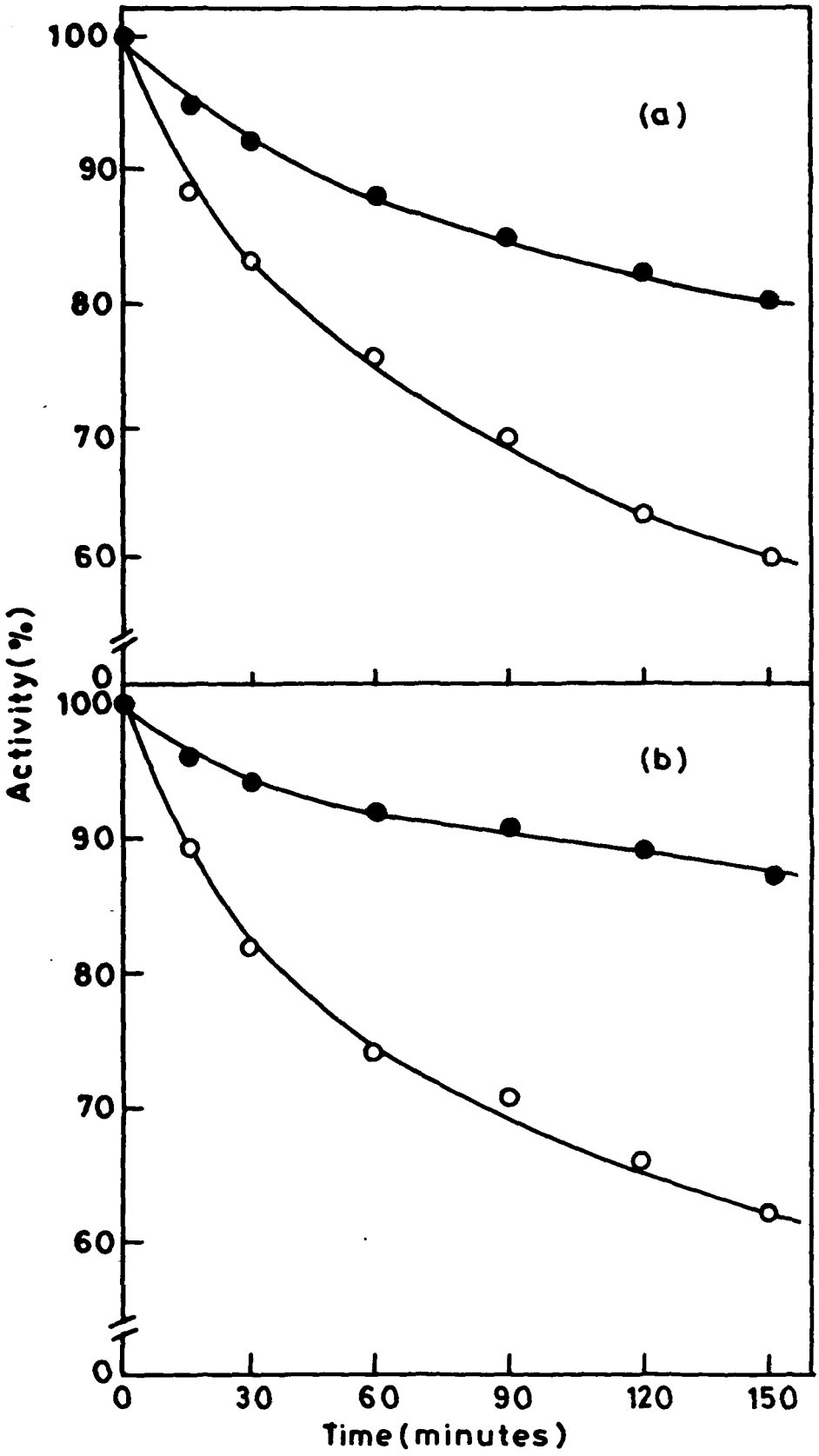

100
90
80
70
60
0
(a)
100
90
80
70
60
0
(b)
Activity(%)
0
30
60
90
120
150
Time(minutes)

Fig. 19 Denaturant stability of soluble and insolubilized papain preparations (B).

Stability of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), towards denaturants was determined by incubating these preparations in 4 M urea at 37°C for the indicated durations, and activity was determined under standard conditions.

(Δ) soluble papain

(▲) insolubilized papain

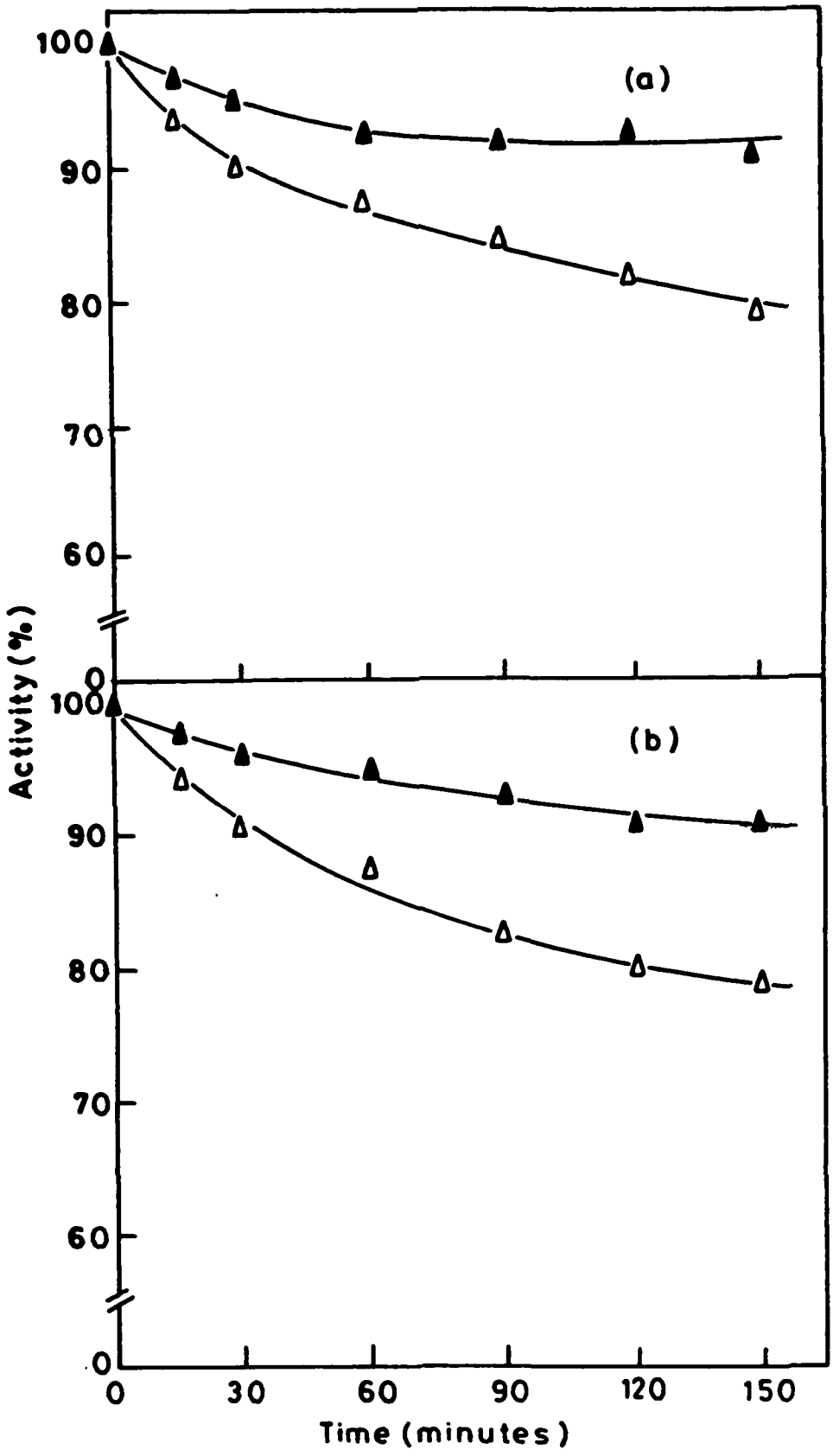

100
90
80
70
60
0
(a)
100
90
80
70
60
0
(b)
Activity (%)
0
30
60
90
120
150
Time (minutes)

Fig. 20 pH dependence of the enzymatic activity of soluble and insolubilized papain preparations (A).

pH dependence of enzyme activity of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), was carried out at 37°C under standard conditions.

(o) soluble papain,

(●) insolubilized papain.

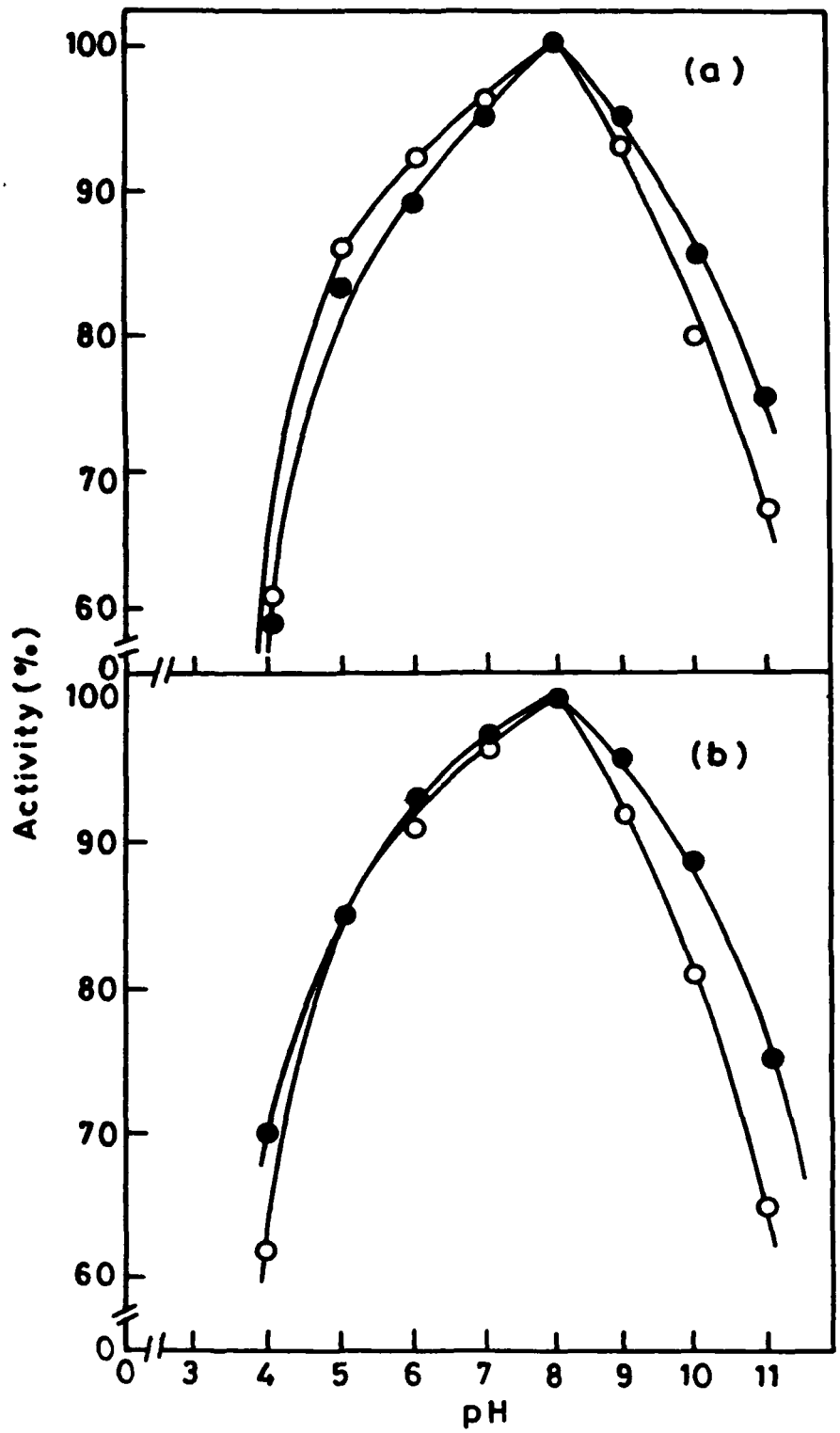
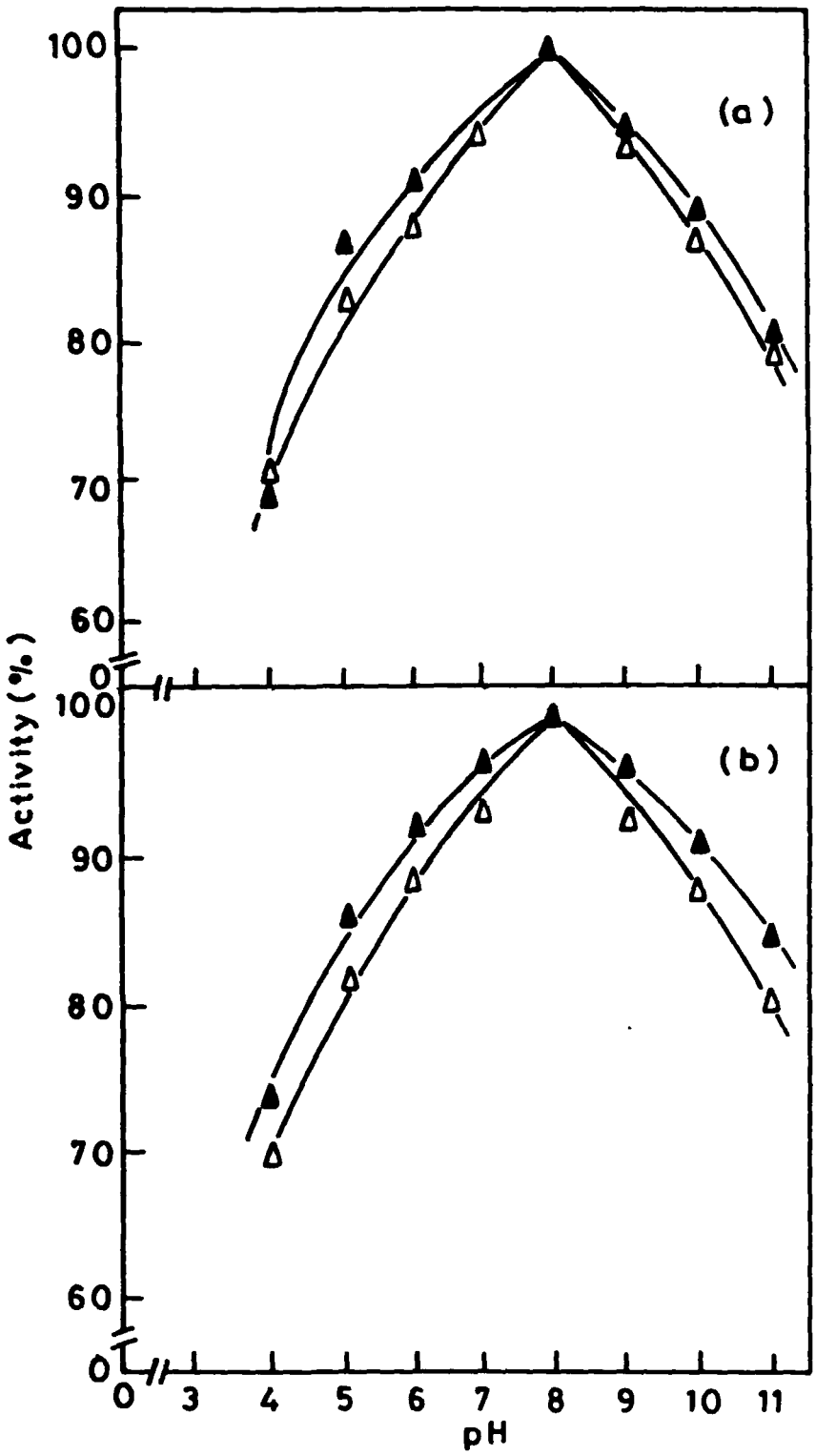

(a)
(b)
100
90
80
70
60
0
Activity (%)
0
3
4
5
6
7
8
9
10
11
pH

Fig. 21 pH dependence of the enzymatic activity of soluble and insolubilized papain preparations (B).

pH dependence of enzyme activity of papain insolubilized by complexing with antipapain antiserum (a) or by complexing with IgG (b), was carried out at 37°C under standard conditions.

(Δ) soluble papain,

(▲) insolubilized papain.


100
90
80
70
60
0
(a)
100
90
80
70
60
0
(b)
Activity (%)
0
3
4
5
6
7
8
9
10
11
pH

(iv) Effect of Substrate Concentration : The K_m and V_{max} values of native and immunocomplex (B) of papain obtained by precipitation with antiserum (a) and IgG (b) fractions were determined by double reciprocal plots using BAPNA as substrate (Fig. 22). The values obtained are given in Table V. The observed K_m values of both immunocomplexes used for these studies were different from the soluble papain.

(v) Effect of Metal Ions : Effect of some metal ions (Cd^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}) on soluble and immunocomplex (B) prepared from IgG of papain was observed in the presence of fixed amount of EDTA. These metal ions are inhibitory for papain while EDTA brings about its reactivation. As shown in Table VI at the lower concentration of these metal ions in the presence of 9 mM EDTA, complete reactivation of enzyme was observed by both insolubilized papain and its soluble counterpart. However, as the concentration of metal ions increased from 1 mM to 100 mM, the reactivation of the enzyme was not so pronounced at higher metal ion concentration for soluble papain. At 100 mM concentration of Cd^{2+}, 32% activity of soluble and 74% of insolubilized preparations were retained and similar pattern was observed by other metal ions of the same concentration used in the experiment.

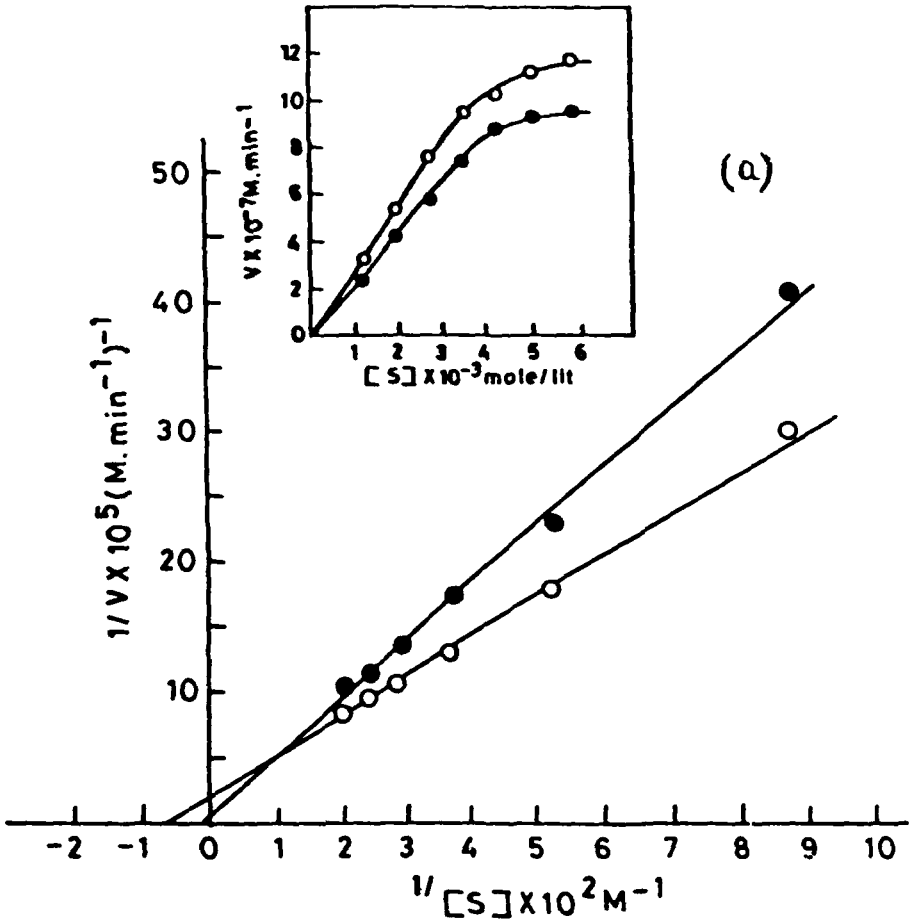
(vi) Storage Stability : The insolubilized complexes (A and B) of papain prepared with IgG could be stored for longer durations without any substantial

Fig. 22 Effect of substrate concentration on the peptidase activity of soluble and insolubilized papain preparations (B).

Approximately 110 units of soluble and insolubilized papain preparations (B) complexed with antipapain antiserum (a) or complexed with IgG (b) was incubated in a series of tubes containing varying concentrations of substrate (BAPNA) in the standard assay mixture for 30 minutes at 37°C and extent of hydrolysis of BAPNA was determined. Reciprocal concentration of velocity were plotted as a function of the reciprocal of substrate to obtain a characteristic Line Weaver Burk plot. The lines were extrapolated to cut the X-axis in order to obtain the volume of $1/K_m$ from which the K_m value was determined.

(o) soluble papain

(●) insolubilized papain


(a)
1/V X 10^5 (M.min^-1)^-1
1/[S] X 10^2 M^-1
V X 10^-7 M.min^-1
[S] X 10^-3 mole/lit

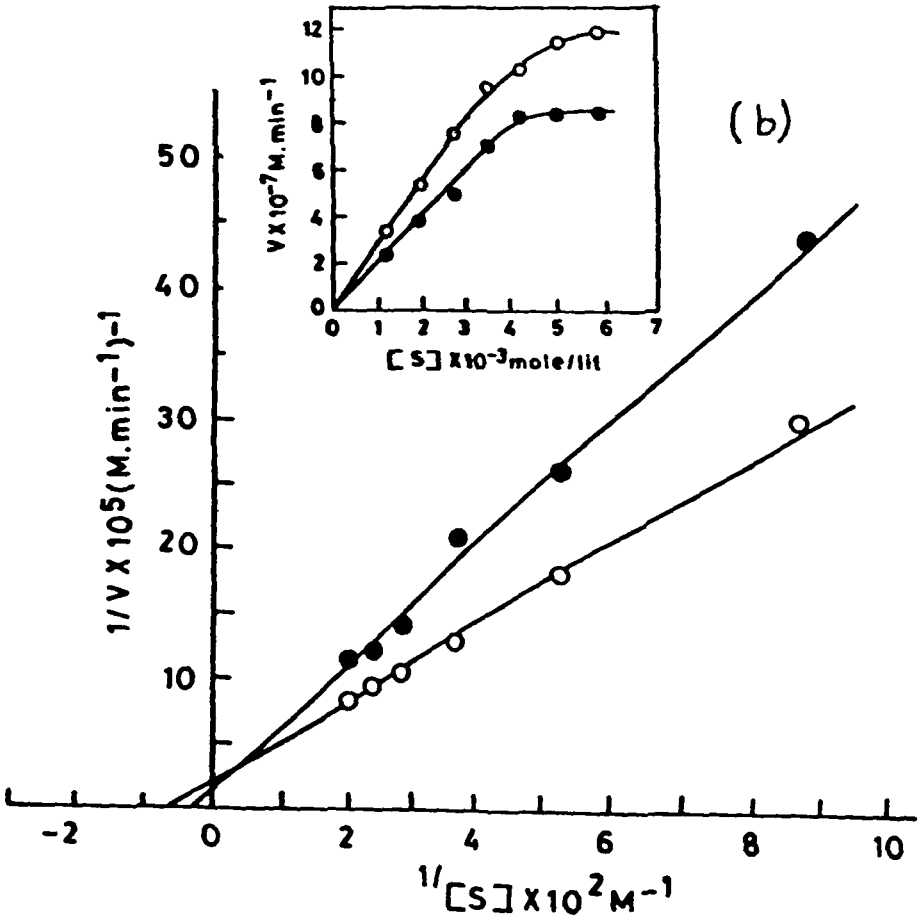

(b)
1/V X 10^5 (M.min^-1)^-1
1/[S] X 10^2 M^-1
V X 10^-7 M.min^-1
[S] X 10^-3 mole/lit

Table V. **K_m and Vmax values of papain with synthetic substrate (BAPNA).**

Appropriate amounts of soluble and insolubilized papain were incubated with varying amounts of substrate. K_m and Vmax were determined graphically from the Line Weaver Burk plots of the data.

Papain Preparations	**Km (M)**	**Vmax ($M.min^{-1}$)**
Soluble papain	14.3×10^{-3}	4.72×10^{-6}
*Preparation (B) complexed with antipapain antiserum	50.0×10^{-3}	22.2×10^{-6}
*Preparation (B) complexed with IgG	33.3×10^{-3}	6.85×10^{-6}

*Described in the text.

Table VI. Effect of metal ions in the presence of EDTA on the activity of soluble and insolubilized papain.

116 units of soluble [S] and 95 units of insolubilized [I] preparation (B) of papain were incubated with different concentrations of metal ions in the presence of 9 mM EDTA for 1 hr. Activity was determined by the described procedure.

Metal ion (mM)	Papain Activity (%)							
	Cd^{2+}		Fe^{2+}		Cu^{2+}		Zn^{2+}	
	[S]	[I]	[S]	[I]	[S]	[I]	[S]	[I]
0	100	100	100	100	100	100	100	100
1	96	97	88	97	74	79	97	98
10	85	96	63	68	47	67	93	95
20	79	95	51	58	29	57	82	93
50	45	87	27	49	22	50	31	73
100	32	74	21	44	15	42	24	67

loss in the enzyme activity. As evident from Table VII the soluble counterparts of preparations (A) and (B) lost 41% and 33% activities respectively, when stored at 4°C for 60 days, while the immunocomplex (A) and (B) retained 75% and 80% activities respectively for the same duration.

V. IMMOBILIZATION OF PAPAIN ON IMMUNOAFFINITY SUPPORT

Two insoluble complexes of papain, (A) and (B) with low and high antibody to enzyme ratios were prepared with excellent retention of activity and stability properties. Some difficulties may encounter with regard to the flow rates especially during the operation of relatively large columns. It was, therefore, considered worthwhile to investigate the properties of papain immobilized on beaded Seralose-4B via an antibody spacer which exhibit excellent flow properties.

Influence of Enzyme Concentration on Immobilization : As shown in the earlier section the immunocomplex (B) having high antibody to enzyme ratio was superior in activity and stability over the immunocomplex (A) where antibody to enzyme ratio was low. In both the preparations the fixed amount of antibody was used with two different concentrations of papain which was the major factor for low and high antibody to enzyme ratio. During the immobilization of papain on the immunoaffinity support, consideration has been

Table VII. Storage stability of soluble and insolubilized papain at 4°C.

Soluble and insolubilized preparations of papain were stored at 4°C and aliquot was withdrawn at the indicated period of time and activity was determined. The day preparation was kept, was considered as 0 day and percent remaining activity of the subsequent days was calculated from that value.

Storage time (days)	Percent remaining activity			
	Preparation (A)		Preparation (B)	
	Soluble	Insolubilized	Soluble	Insolubilized
0	100	100	100	100
15	82	92	87	94
30	70	86	78	89
45	64	80	71	84
60	59	75	67	80

made to see the influence of papain concentration on the activity and stability properties. Pure papain is fairly expensive enzyme which would add to the cost of matrix obtained after immobilization. Optimal utilization of immunoaffinity adsorbed enzyme is, therefore, essential. In view of this, immobilization of papain was investigated as a function of enzyme concentration bound to antibody loaded matrix. Tables VIII and IX summarize the effect of two concentrations of papain immobilized on antiserum/IgG loaded matrices. The preparations were designated as 'high' and 'low' enzyme bound matrices. 5 mg antisera/IgG were immobilized on each gram of Seralose-4B. In order to obtain 'high' enzyme bound matrix, 27,360 and 33,360 units of papain were adsorbed on antiserum as well as IgG loaded matrices (Table VIII) while 'low' enzyme bound matrices were prepared by immobilizing 9360 and 12840 units of papain on antiserum/IgG coupled Seralose-4B (Table IX). As shown in Table the η values were 0.38 and 0.42, when 'high' concentration of papain was loaded on antiserum as well as IgG bound Saralose-4B. η is an effectiveness factor, a ratio of measured to bound activity and gives information on the accessibility of an enzyme to the substrate. A drastic increase in the η values were observed at 'low' enzyme concentration used during immobilization on the antiserum/IgG bound matrices. These values were 0.94 and 0.96 for antiserum and IgG bound matrices respectively.

Table VIII. Immobilization of papain on antibody support.

Each value represents the average result of atleast three experiments performed in duplicate. In order to obtain 'theoretical' binding, units unbound and those in the washing were substracted from the added units. An appropriate aliquot of the immobilized preparation was assayed to obtain the 'actual' activity. η values represent the ratios of 'actual' and 'theoretical' activity of papain immobilized on antipapain antiserum/IgG support.

	Units added	Units in washes	Immobilized Activity (Units)		η value
			Theoretical (a)	Actual (b)	b/a
Preparation (H_1)	3,78,750	3,05,550	73200	27360	0.38
Preparation (H_2)	3,80,000	3,00,975	79025	33360	0.42

H_1 -'High' papain concentration coupled to antiserum bound Seralose-4B

H_2 - 'High' papain concentration coupled to IgG bound Seralose-4B.

Table IX. Immobilization of papain on antibody support.

Each value represents the average result of atleast three experiments performed in duplicate. In order to obtain 'theoretical' binding, units unbound and those in the washing were substracted from the added units. An appropriate aliquot of the immobilized preparation was assayed to obtain the 'actual' activity. η values represent the ratios of 'actual' and 'theoretical' activity of papain immobilized on antipapain antiserum/IgG support.

	Units added	Units in washes	Immobilized Activity (Units)		η value b/a
			Theoretical (a)	Actual (b)	
Preparation (L_1)	42,500	32,550	9950	9360	0.94
Preparation (L_2)	41,500	28,100	13400	12840	0.96

L_1 - 'Low' papain concentration coupled to antiserum bound Seralose-4B.

L_2 - 'Low' papain concentration coupled to IgG bound Seralose-4B.

VI. STUDIES ON ANTIBODY MEDIATED IMMOBILIZED PAPAIN

The stability of various immobilized papain preparations on Seralose-4B against heat and urea denaturation were investigated. The stability of 'low' enzyme bound matrix was very prominantly high in comparison to the 'high' enzyme bound matrix. A remarkable finding was also noticed with the soluble preparation of papain, a counterpart of 'low' enzyme bound matrix, in terms of its higher stability.

(i) Effect of Temperature : The effect of incubation at 75°C on 'high' enzyme bound to antiserum and IgG mediated matrices is shown in Fig. 23 (a and b). Both immobilized preparations exhibited marked enhancement in thermal stability as compared to the native enzyme. The immobilized preparations (H_1) and (H_2) retained 60% and 70% activities respectively after 2 hrs incubation at 75°C, while their soluble counterpart retained only 25% activity for the same duration. A different pattern of thermal denaturation was observed in the immobilized as well as soluble preparations of papain by decreasing the enzyme concentration. The results of (L_1) and (L_2) immobilized preparations alongwith their soluble counterparts are shown in Fig. 24 (a and b). Only 10% activity was lost by the immobilized preparation after incubation at 75°C for 2 hrs. At the same time the soluble papain having low concentration was also stable and retained 80% activity under similar conditions.

Fig. 23 Thermal inactivation of soluble and immobilized papain preparations (H_1 & H_2).

The thermal stability of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), was determined by incubating these preparations for various durations at 75°C. Papain activity was determined at the end of treatment under standard conditions.

(o) soluble papain

(●) immobilized papain

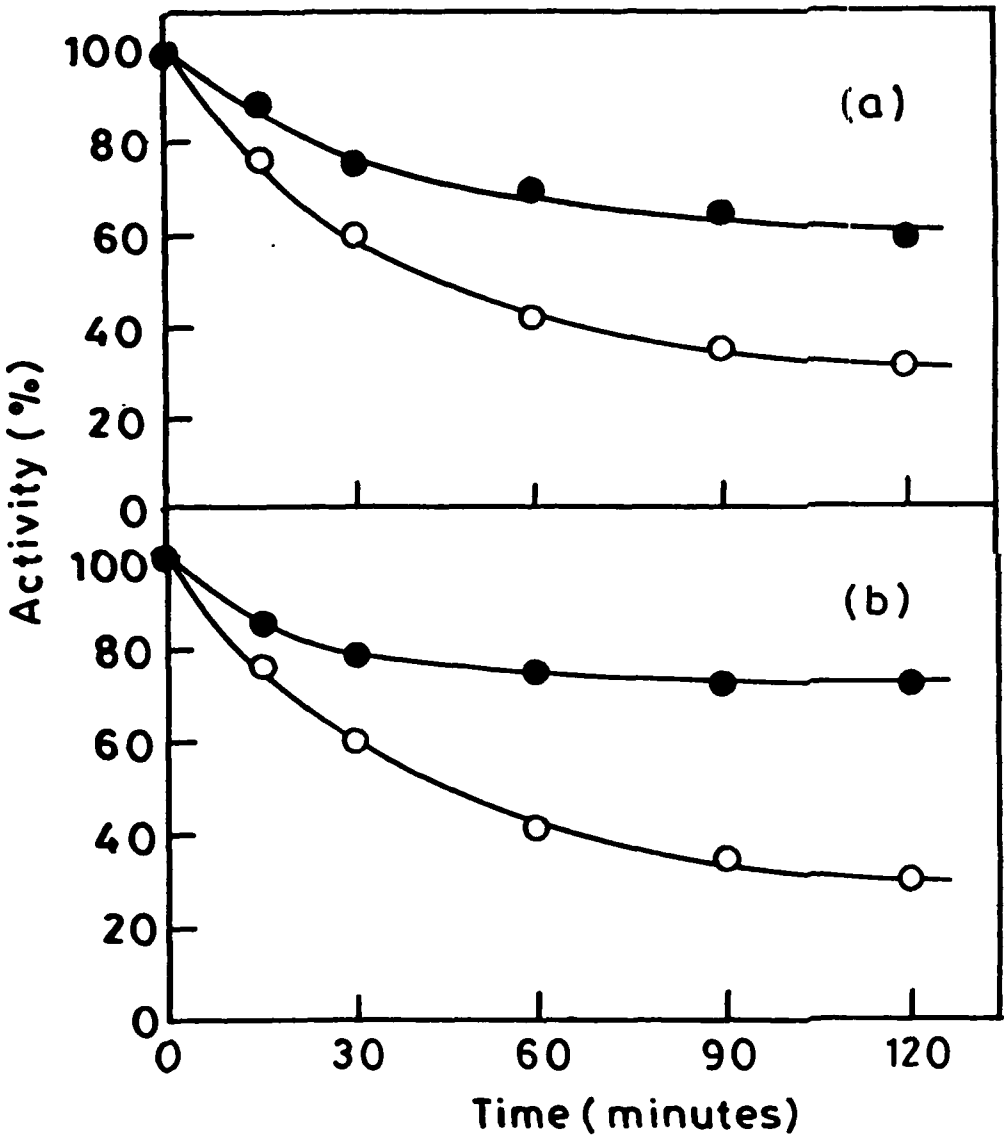

100
80
60
40
20
0
(a)
100
80
60
40
20
0
(b)
Activity (%)
0
30
60
90
120
Time (minutes)

Fig. 24 Thermal inactivation of soluble and immobilized papain preparations (L_1 & L_2).

The thermal stability of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), was determined by incubating these preparations for various durations at 75°C. Papain activity was determined at the end of treatment under standard conditions.

(Δ) soluble papain

(▲) immobilized papain

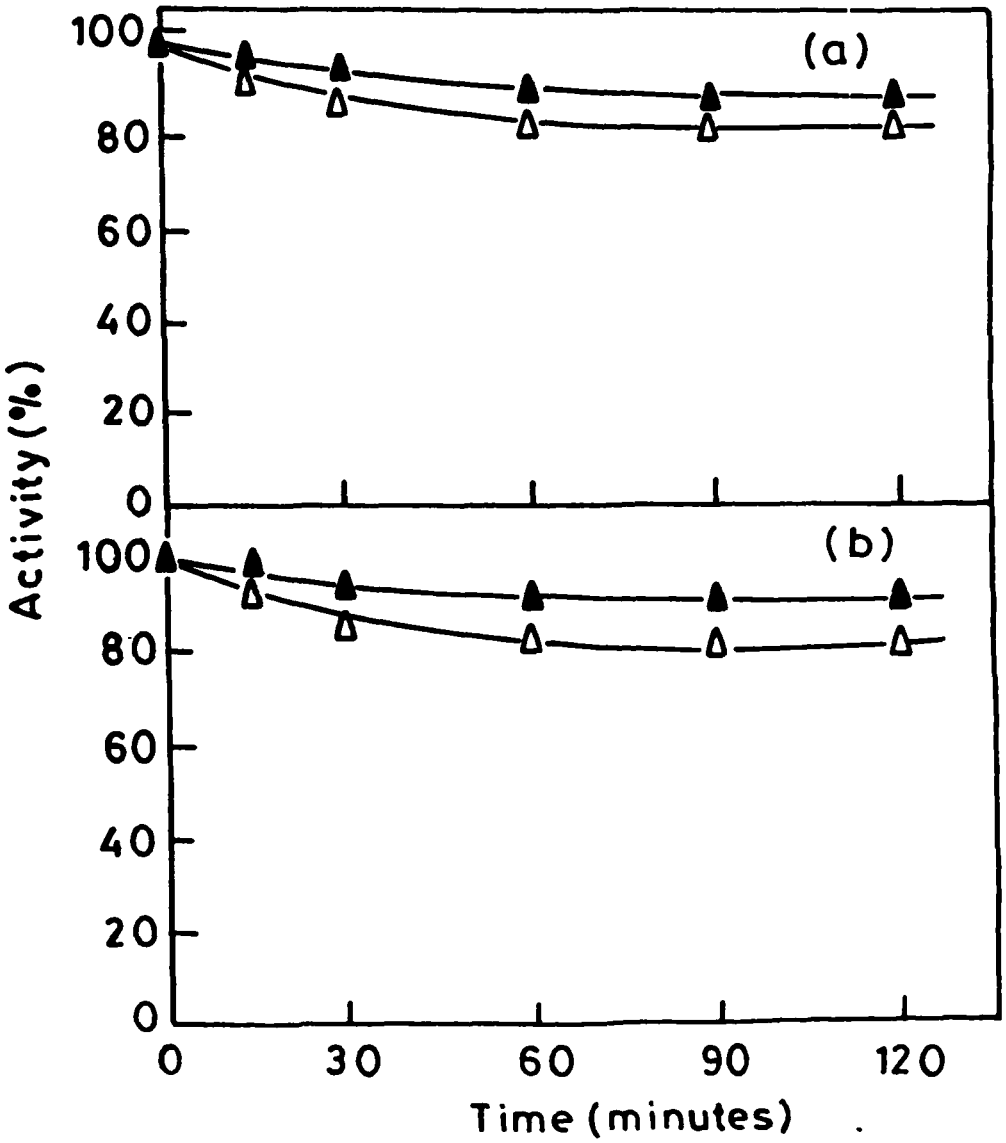

100
80
60
40
20
0
(a)
100
80
60
40
20
0
(b)
Activity (%)
0
30
60
90
120
Time (minutes)

Fig. 25 and 26 (a & b) show the thermostability curves of papain in its native and immobilized forms. At the maximum temperature of 90°C, the 'high' enzyme loaded to antiserum and IgG mediated matrices retained 55 and 65% activities respectively. The soluble papain taken at this concentration retained only 35% activity after incubation for 30 minutes. Similar trend but higher stability for 'low' enzyme concentration bound matrices and their soluble counterparts was achieved under the same conditions. More than 70% activity of the soluble papain at its low concentration was obtained which was higher than the immobilized preparation of Fig. 25 where more enzyme was coupled to its specific antibody support.

(ii) Effect of Urea : The immobilized papain preparations and their respective soluble forms of enzyme were also tested against the denaturation in the presence of 4 M urea. The results are shown in Fig. 27 and 28 (a & b) with the similar pattern of stability as was observed against the heat denaturation. The soluble and matrix bound papain at its 'low' concentration was more resistant to urea denaturation followed by the immobilized preparation having 'high' enzyme concentration.

(iii) Effect of pH : The 'high' concentration of papain in soluble and immobilized forms bound to antiserum or IgG support did not show any alteration in the pH activity profile compared to soluble enzyme (Fig. 29 (a & b)). Maximum activity was observed at pH 8. There was no significant

Fig. 25 Effect of pretreatment at various temperatures on soluble and immobilized papain preparations (H_1 & H_2).

Thermal inactivation of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), was determined by incubating these preparations at various temperatures for 30 minutes, cooled and activity was determined under standard conditions.

(o) soluble papain

(●) immobilized papain

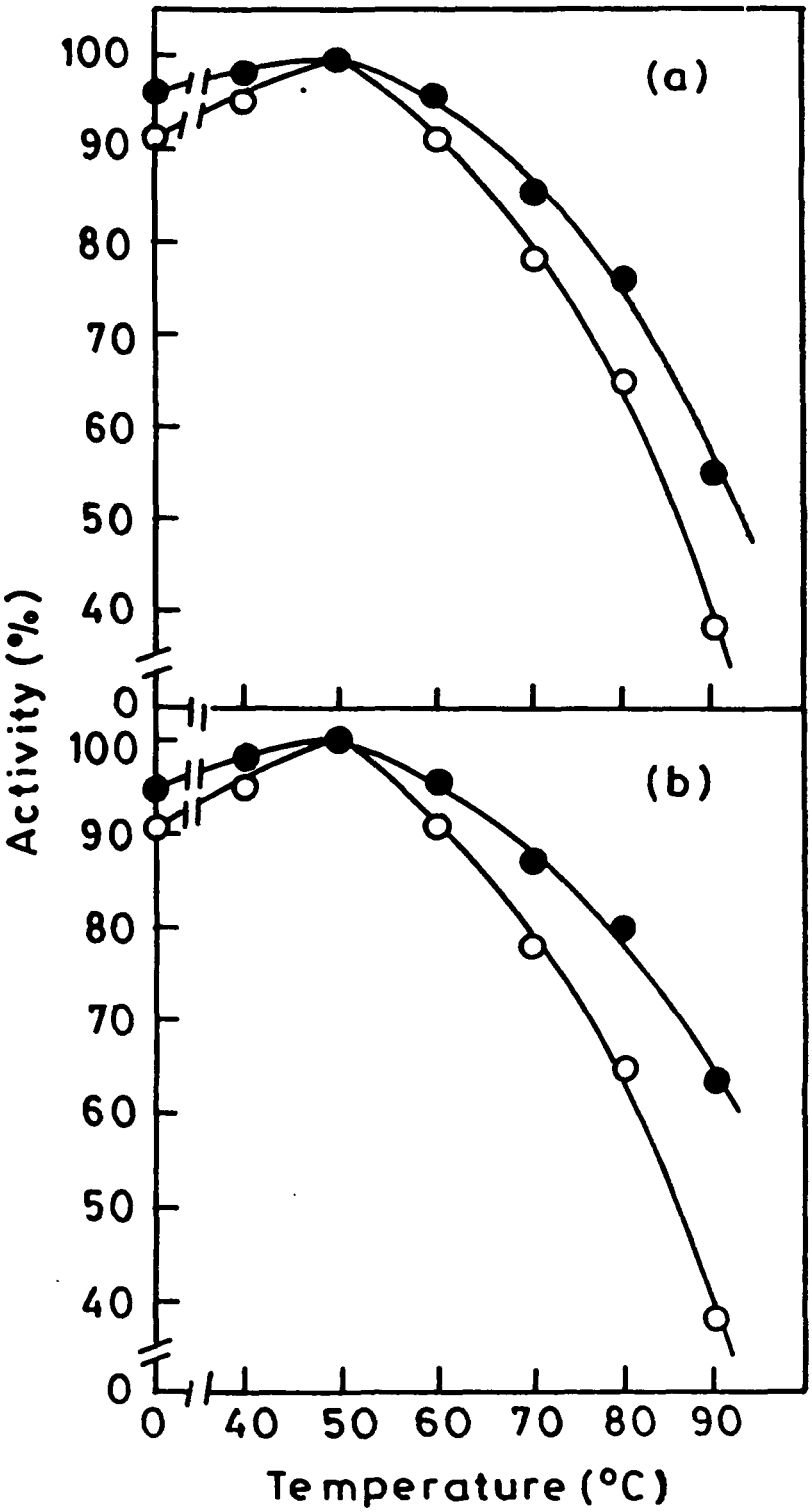

(a)
(b)
100
90
80
70
60
50
40
0
Activity (%)
0
40
50
60
70
80
90
Temperature (°C)

Fig. 26 Effect of pretreatment at various temperatures on soluble and immobilized papain preparations (L_1 & L_2).

Thermal inactivation of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), was determined by incubating these preparations at various temperatures for 30 minutes, cooled and activity was determined under standard conditions.

(Δ) soluble papain

(▲) immobilized papain

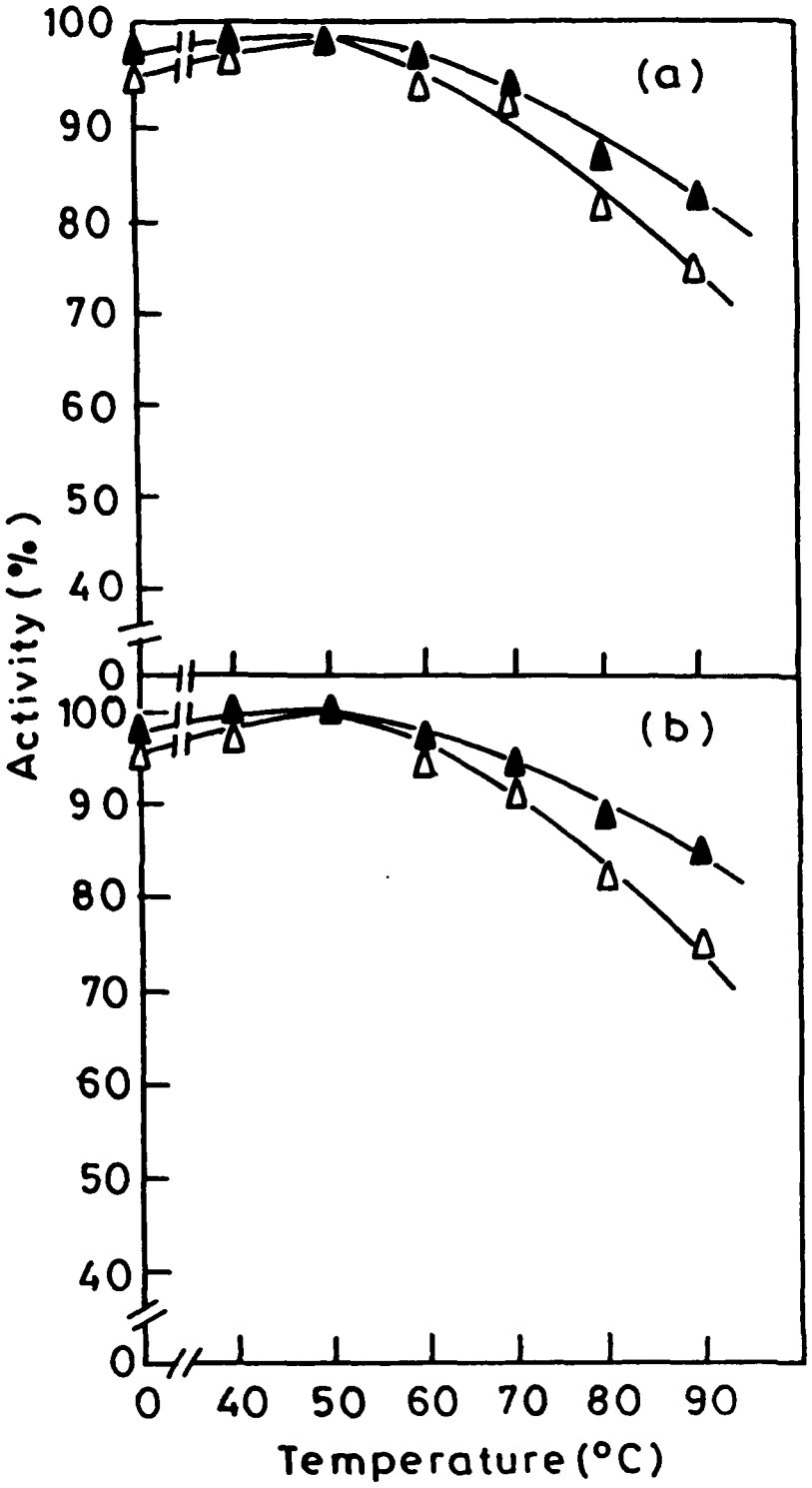

100
90
80
70
60
50
40
0
(a)
100
90
80
70
60
50
40
0
(b)
Activity (%)
0
40
50
60
70
80
90
Temperature (°C)

Fig. 27 Denaturant stability of soluble and immobilized papain preparations (H_1 & H_2).

Stability of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), towards denaturants was determined by incubating these preparations in 4 M urea at 37°C for the indicated durations, and activity was determined under standard conditions.

(o) soluble papain

(●) immobilized papain

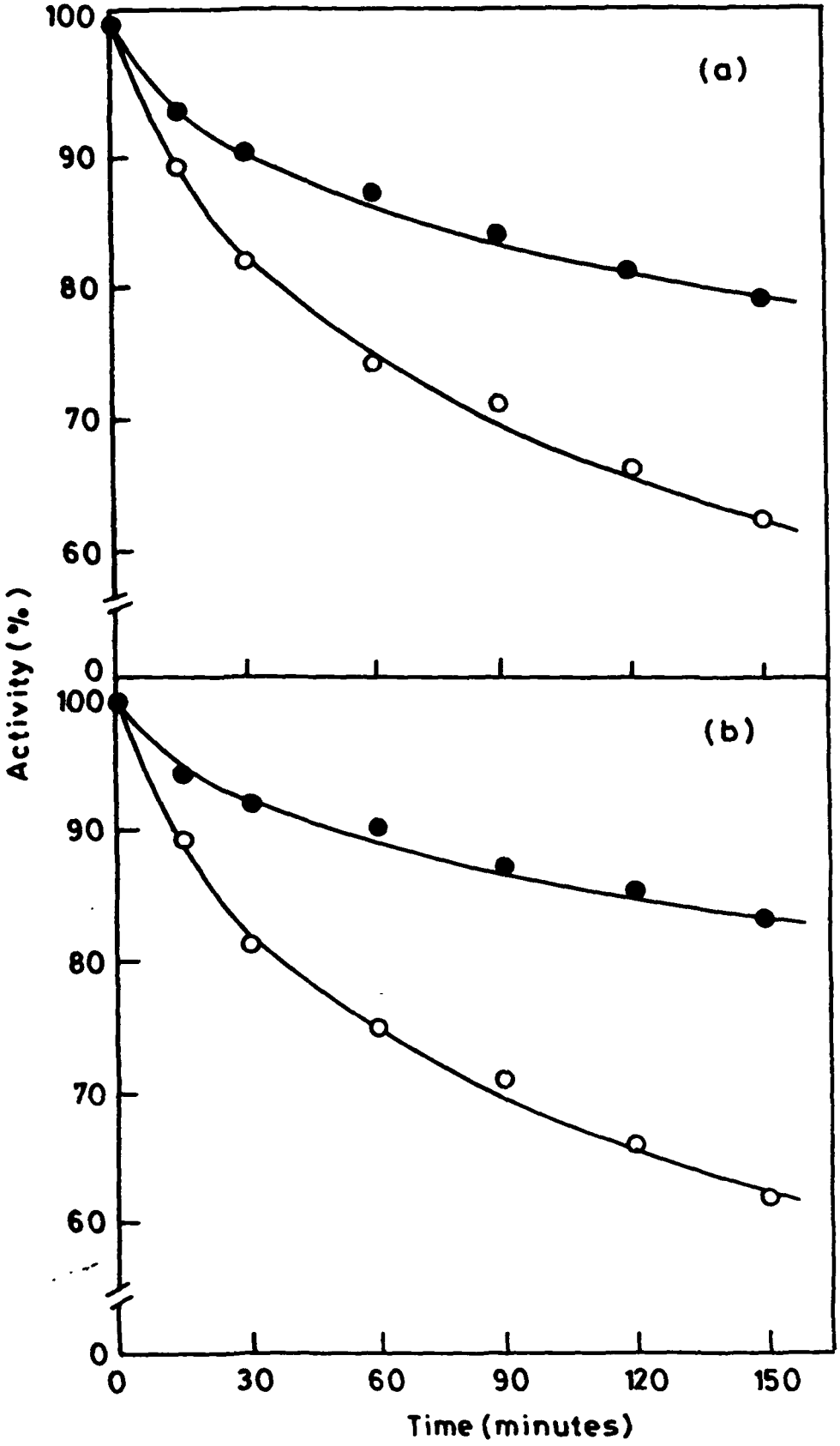

(a)
(b)
100
90
80
70
60
0
Activity (%)
0
30
60
90
120
150
Time (minutes)

Fig. 28 Denaturant stability of soluble and immobilized papain preparations (L_1 & L_2).

Stability of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), towards denaturants was determined by incubating these preparations in 4 M urea at 37°C for the indicated durations, and activity was determined under standard conditions.

(Δ) soluble papain

(▲) immobilized papain

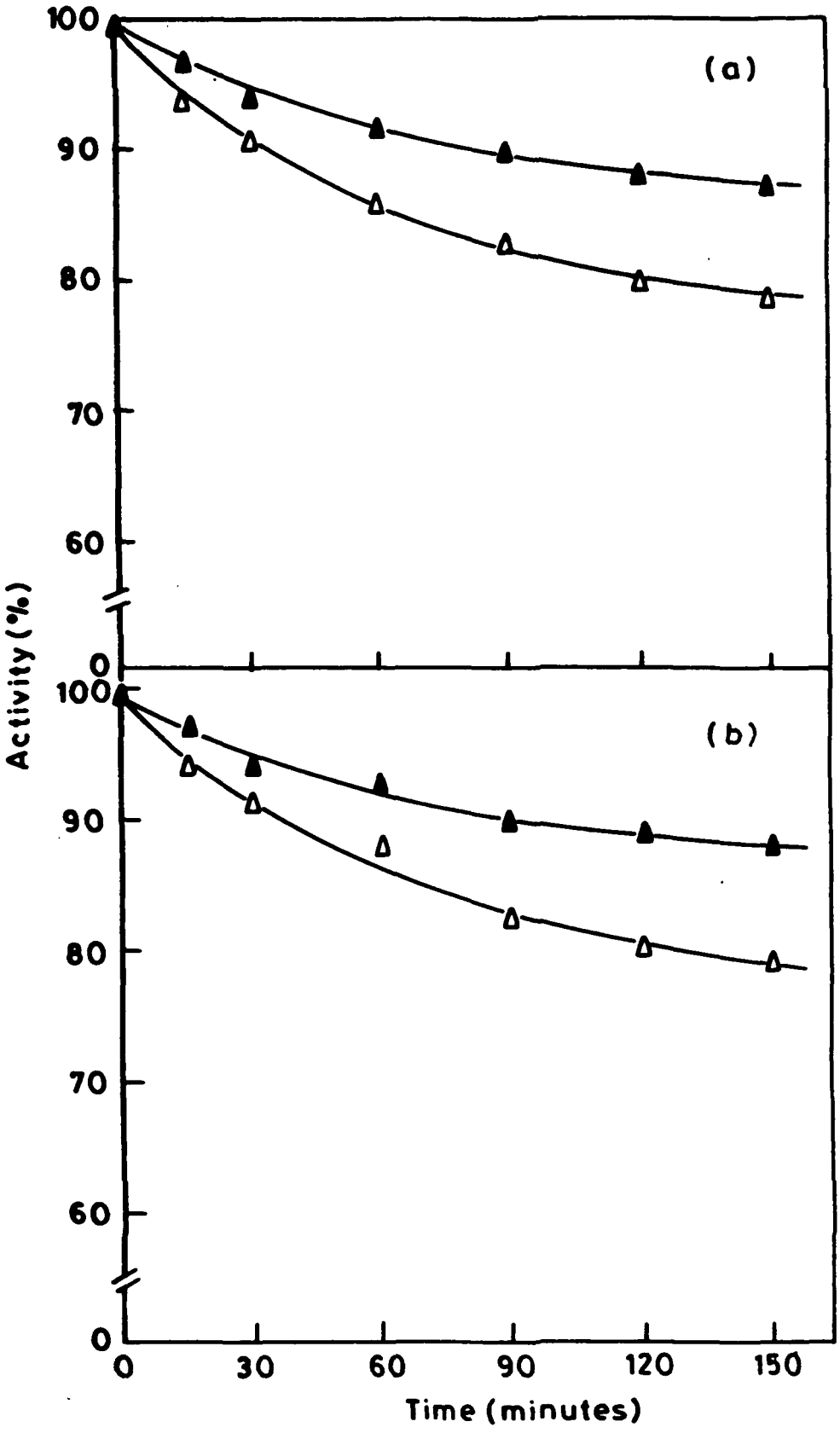
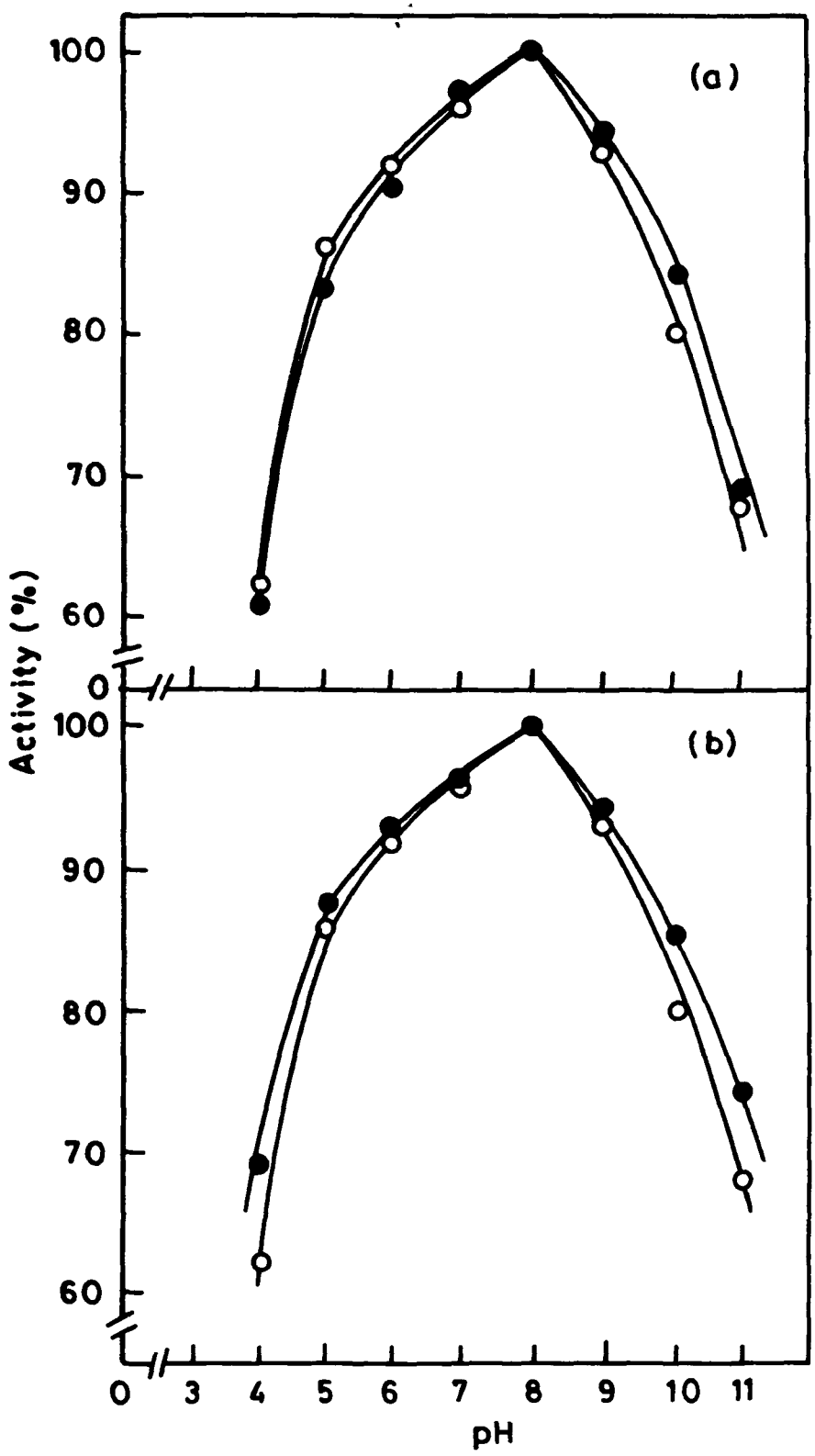

100
90
80
70
60
0
(a)
100
90
80
70
60
0
(b)
Activity (%)
0
30
60
90
120
150
Time (minutes)

Fig. 29 pH dependence of the enzymatic activity of soluble and immobilized papain preparations (H_1 & H_2).

pH dependence of enzyme activity of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), was carried out at 37°C under standard conditions.

(o) soluble papain

(●) immobilized papain


(a)
(b)
100
90
80
70
60
0
Activity (%)
3
4
5
6
7
8
9
10
11
pH

broadening of the pH activity curve for the immobilized enzyme. The papain at its 'low' concentration in soluble and immobilized form also exhibited similar results with maximum activity at pH 8 (Fig. 30 (a & b)).

(iv) Effect of Substrate Concentration : The papain at its 'low' concentration was used to see the effect of substrate concentration on the enzyme activity. The papain activity was measured as a function of substrate (BAPNA) concentration and a typical Michaelis Menton behaviour was observed both in the soluble and immobilized forms (Fig. 31). The K_m and the Vmax values for soluble, antiserum bound and IgG bound matrices calculated from the plots are shown in Table X. The values of the immobilized preparations were deviated from the soluble one.

VII. DIRECT IMMOBILIZATION OF PAPAIN ON SERALOSE-4B

In our earlier studies, an extended spacer of antibody was used to link the enzyme with the solid support which minimizes steric hindrance by the carrier. Attempts have also been made to couple the enzyme directly on the solid support in order to see the activity and stability behaviour of the immobilized enzyme. Two concentrations of papain designated as 'high' and 'low' used in earlier section were also immobilized on Seralose-4B using cyanogen bromide. As evident from the Table XI, relatively small amount of papain could be immobilized on cyanogen bromide activated Seralose-4B. The

Fig. 30 pH dependence of the enzymatic activity of soluble and immobilized papain preparations (L_1 & L_2).

pH dependence of enzyme activity of papain immobilized on antipapain antiserum bound matrix (a) or IgG bound matrix (b), was carried out at 37°C under standard conditions.

(Δ) soluble papain

(▲) immobilized papain

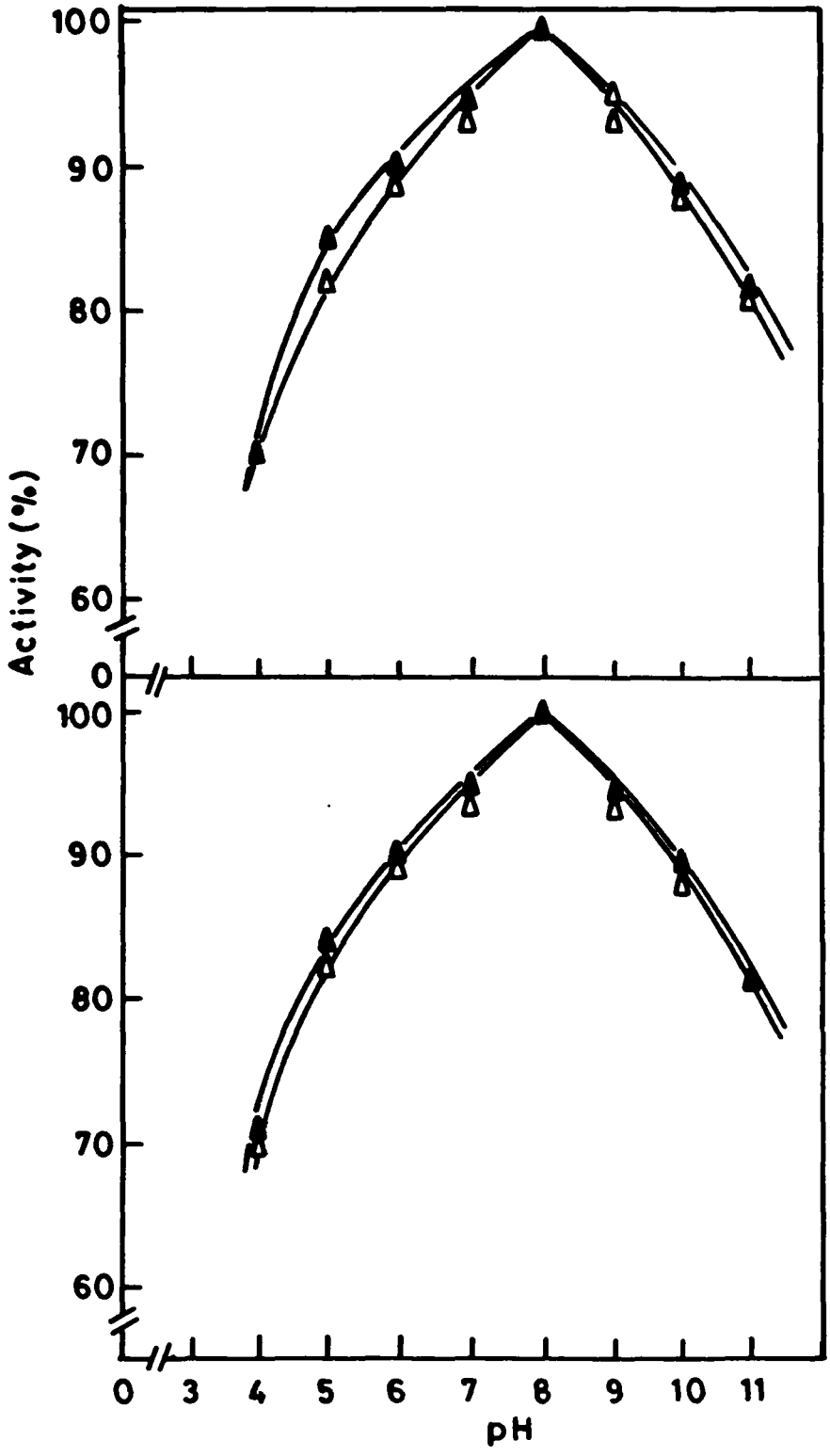
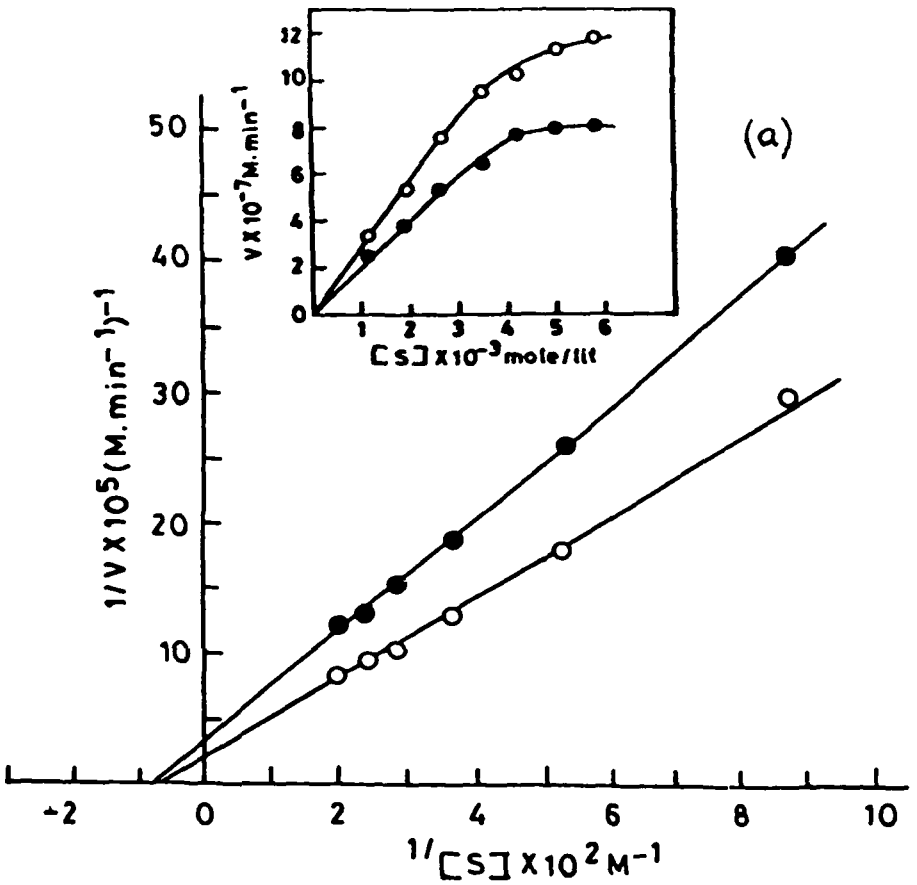

100
90
80
70
60
0
Activity (%)
100
90
80
70
60
0
3
4
5
6
7
8
9
10
11
pH

Fig. 31 Effect of substrate concentration on the peptidase activity of soluble and immobilized papain preparations (L_1 & L_2).

Approximately 100 units of soluble and papain preparations (L_1 & L_2) immobilized on antipapain antiserum bound matrix (a) or immobilized on IgG bound matrix (b) was incubated in a series of tubes containing varying concentrations of substrate (BAPNA) in the standard assay mixture for 30 minutes at 37°C and extent of hydrolysis of BAPNA was determined. Reciprocal concentration of velocity were plotted as a function of the reciprocal of substrate to obtain a characteristic Line Weaver Burk plot. The lines were extra plotted to cut the X-axis in order to obtain the value of $1/K_m$ from which the K_m value was determined.

(o) soluble papain

(●) immobilized papain


(a)
1/V X 10^5 (M.min^-1)^-1
1/[S] X 10^2 M^-1
V X 10^-7 M.min^-1
[S] X 10^-3 mole/lit

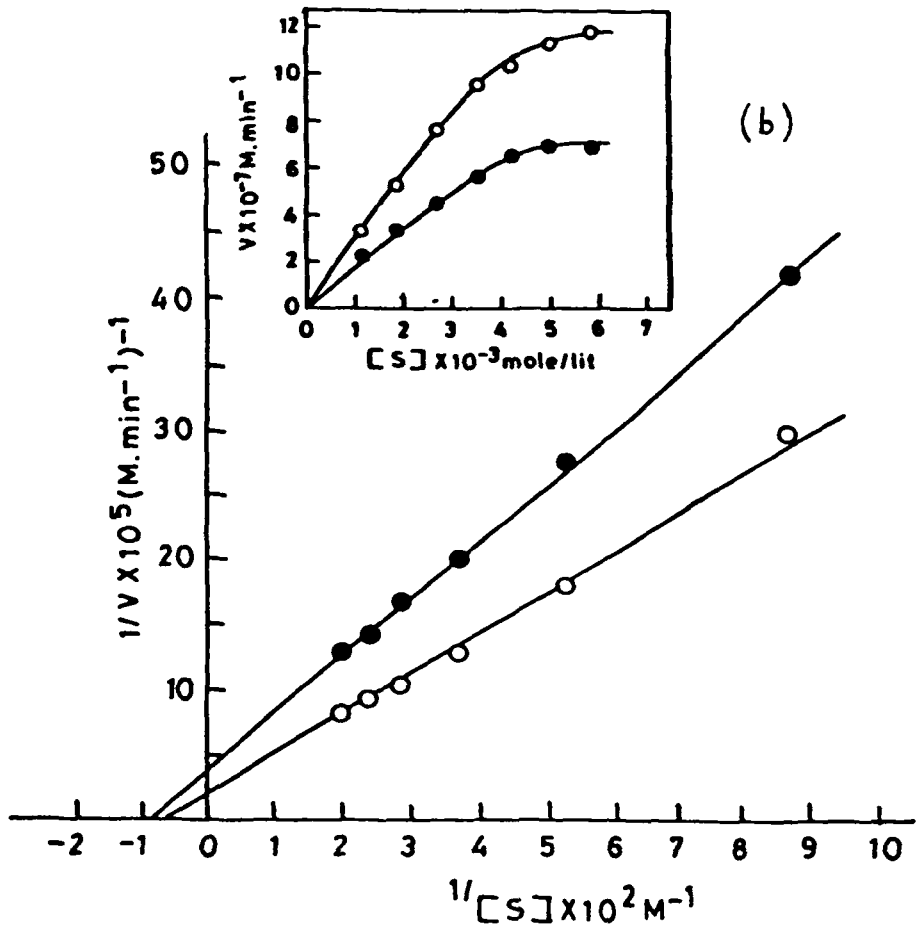

(b)
1/V X 10^5 (M.min^-1)^-1
1/[S] X 10^2 M^-1
V X 10^-7 M.min^-1
[S] X 10^-3 mole/lit

Table X. K_m and Vmax values of papain with synthetic substrate (BAPNA).

Appropriate amounts of soluble and immobilized papain were incubated with varying amounts of substrate. K_m and Vmax were determined graphically from the Line Weaver Burk plots of the data.

Papain Preparations	**K_m (M)**	**Vmax ($M.min^{-1}$)**
Soluble papain	14.3×10^{-3}	4.72×10^{-6}
Preparation (L_1)	12.5×10^{-3}	3.06×10^{-6}
Preparation (L_2)	11.1×10^{-3}	2.53×10^{-6}

L_1 - 'Low' papain concentration coupled to antiserum bound Seralose-4B.

L_2 - 'Low' papain concentration coupled to IgG bound Seralose-4B.

Table XI. Direct immobilization of papain on Seralose-4B.

Each value represents the average results of atleast three experiments performed in duplicate. In order to obtain 'theoretical' binding, units unbound and those in the washing were substracted from the added units. An appropriate aliquot of the immobilized preparation was assayed to obtain the 'actual' activity. η values represent the ratios of 'actual' and 'theoretical' activity of papain immobilized directly on Seralose-4B.

	Units added	Units in washes	Immobilized Activity (Units)		η value
			Theoretical (a)	Actual (b)	b/a
Preparation (H)	81000	15758	65242	18350	0.29
Preparation (L)	37000	10980	26020	20500	0.78

H - 'High' papain concentration used for coupling with Seralose-4B.

L - 'Low' papain concentration used for coupling with Seralose-4B.

η value of 'high' and 'low' enzyme bound preparations were 0.29 and 0.78 respectively (Table XI) clearly suggesting the steric hindrance by the carrier.

VIII. STUDIES ON SERALOSE BOUND PAPAIN

The thermal and urea denaturation of directly immobilized papain at 'high' and 'low' concentration was carried out in a similar manner as described in the earlier sections. A marked increase in thermal stability was observed in the 'low' enzyme bound preparation followed by the 'high' one (Fig. 32 and 33 (a & b)). Similar results were obtained in the urea denaturation studies (Fig. 34 (a & b)). The pH activity profile of native and immobilized preparations of papain was unaffected and no significant broadening was observed in the immobilized preparation (Fig. 35 (a & b)).

Fig. 32 Thermal inactivation of soluble and directly immobilized papain preparations (H&L).

The thermal stability of papain immobilized directly on Seralose- 4B with high (a) or low (b) enzyme concentrations was determined by incubating these preparations for various durations at 75°C. Papain activity was determined at the end of treatment under standard conditions.

(o, Δ) Soluble papain

(●, ▲) Immobilized papain

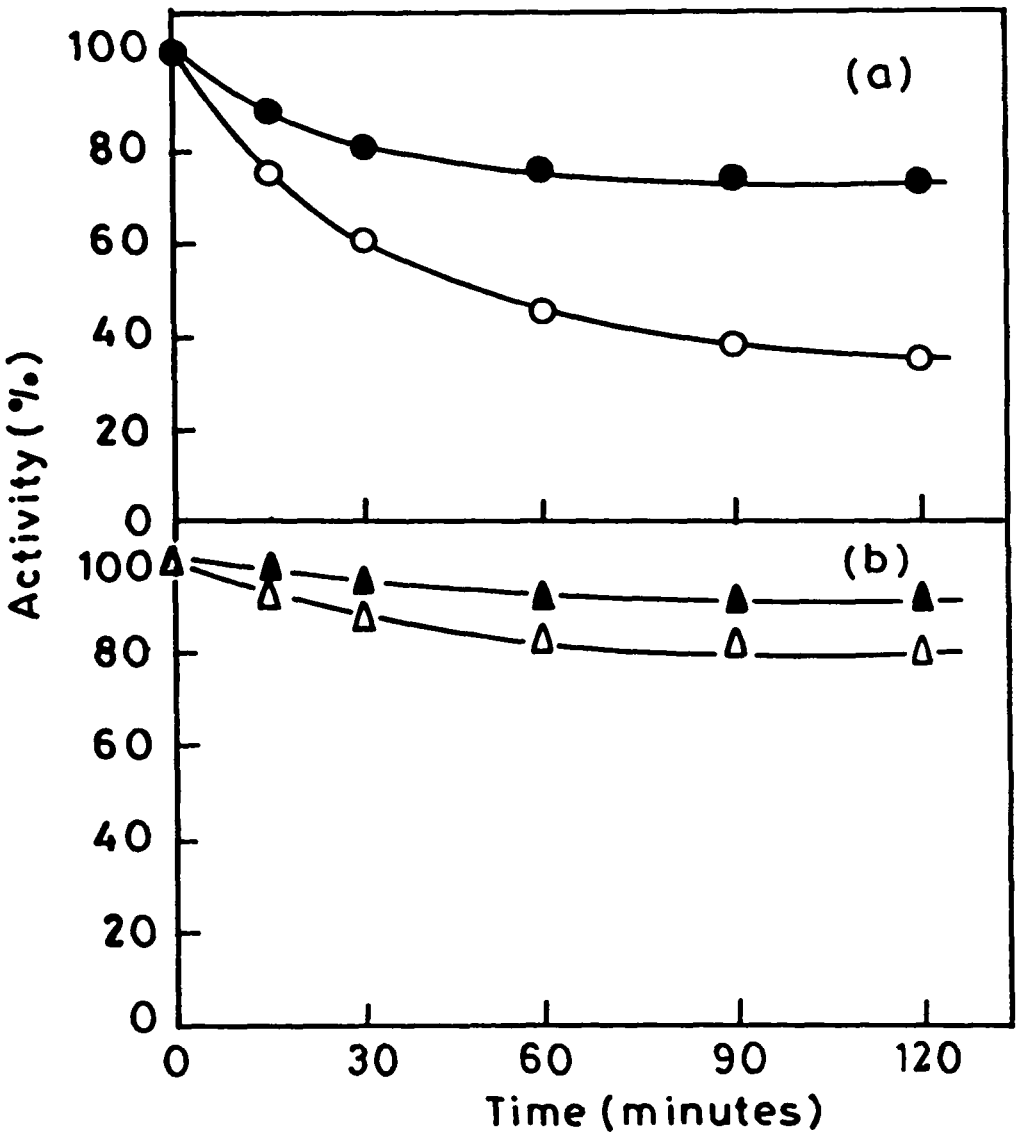

100
80
60
40
20
0
(a)
Activity (%)
100
80
60
40
20
0
(b)
0
30
60
90
120
Time (minutes)

Fig. 33 Effect of pre-treatment at various temperatures on soluble and immobilized papain preparations (H&L).

Thermal inactivation of papain immobilized directly on Seralose- 4B with high (a) or low (b) enzyme concentrations, was determined by incubating these preparations at various temperatures for 30 minutes, cooled and activity was determined under standard conditions.

(o, Δ) Soluble papain

(●, ▲) Immobilized papain

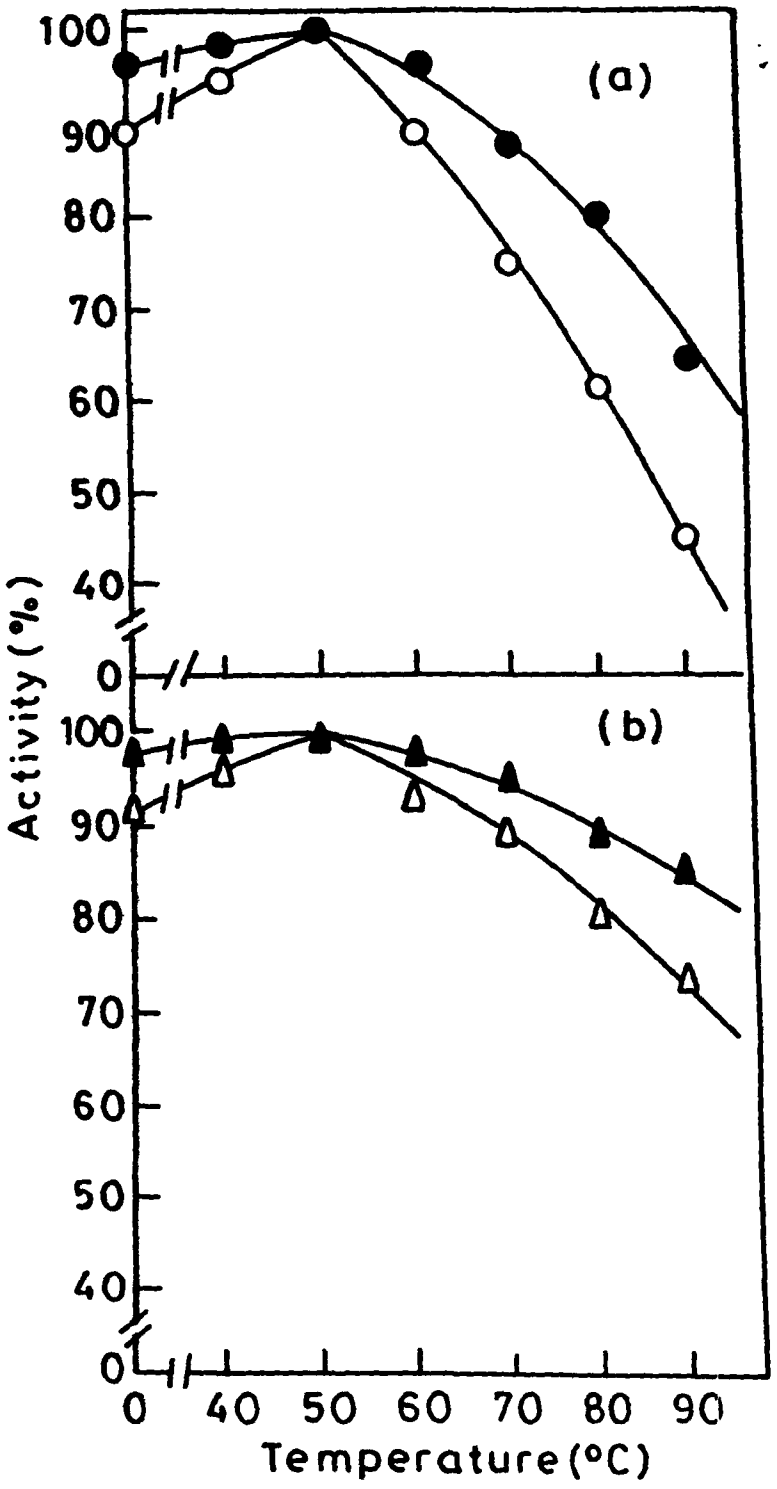

100
90
80
70
60
50
40
0
(a)
100
90
80
70
60
50
40
0
(b)
Activity (%)
0
40
50
60
70
80
90
Temperature (°C)

Fig. 34 Denaturant stability of soluble and immobilized papain preparations (H&L).

Stability of papain directly immobilized on Seralose-4B with high (a) or low (b) enzyme concentrations, towards denaturants was determined by incubating these preparations in 4M urea at 37°C for the indicated durations, and activity was determined under standard conditions.

(o, Δ) Soluble papain

(●, ▲) Immobilized papain

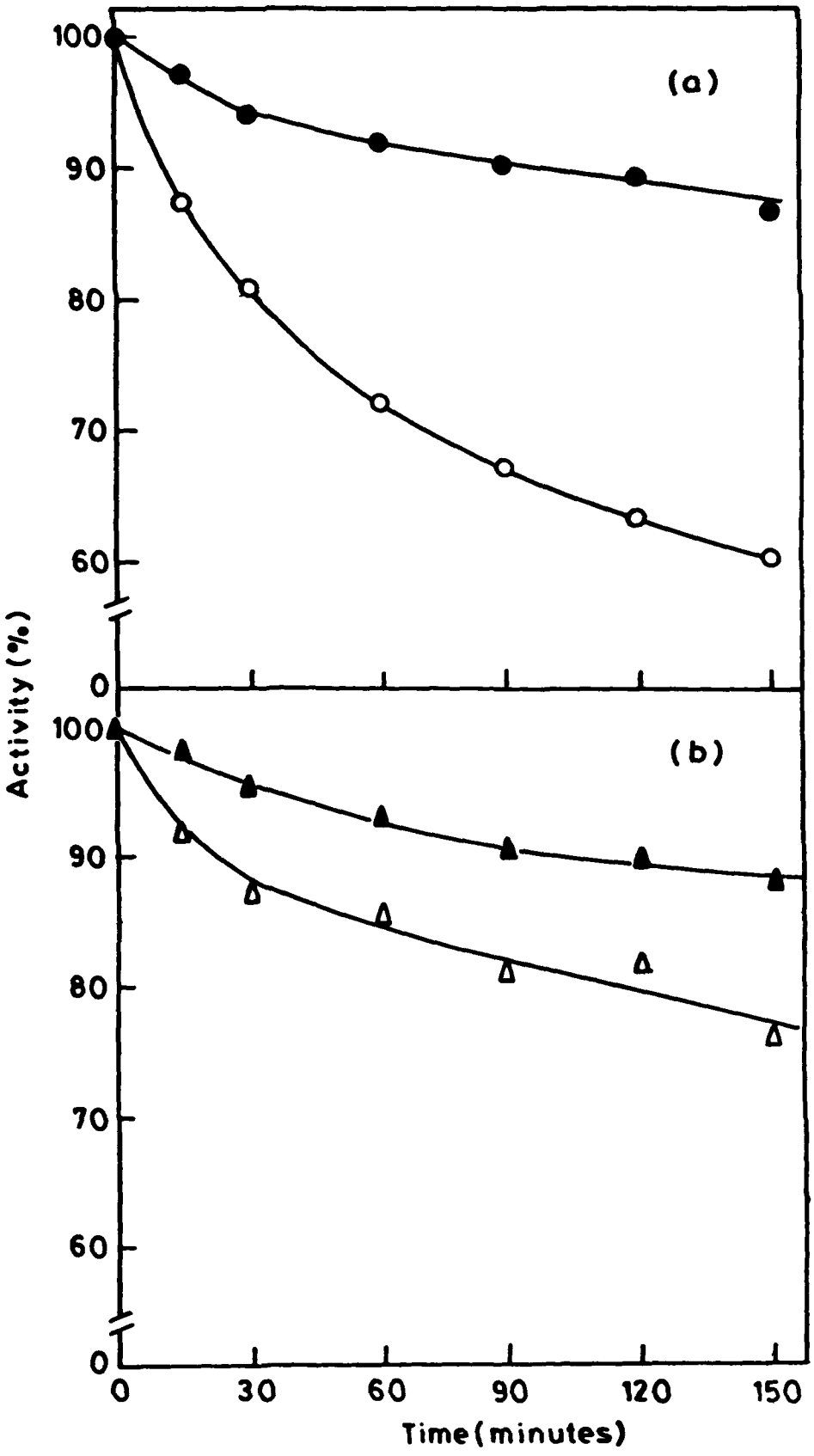
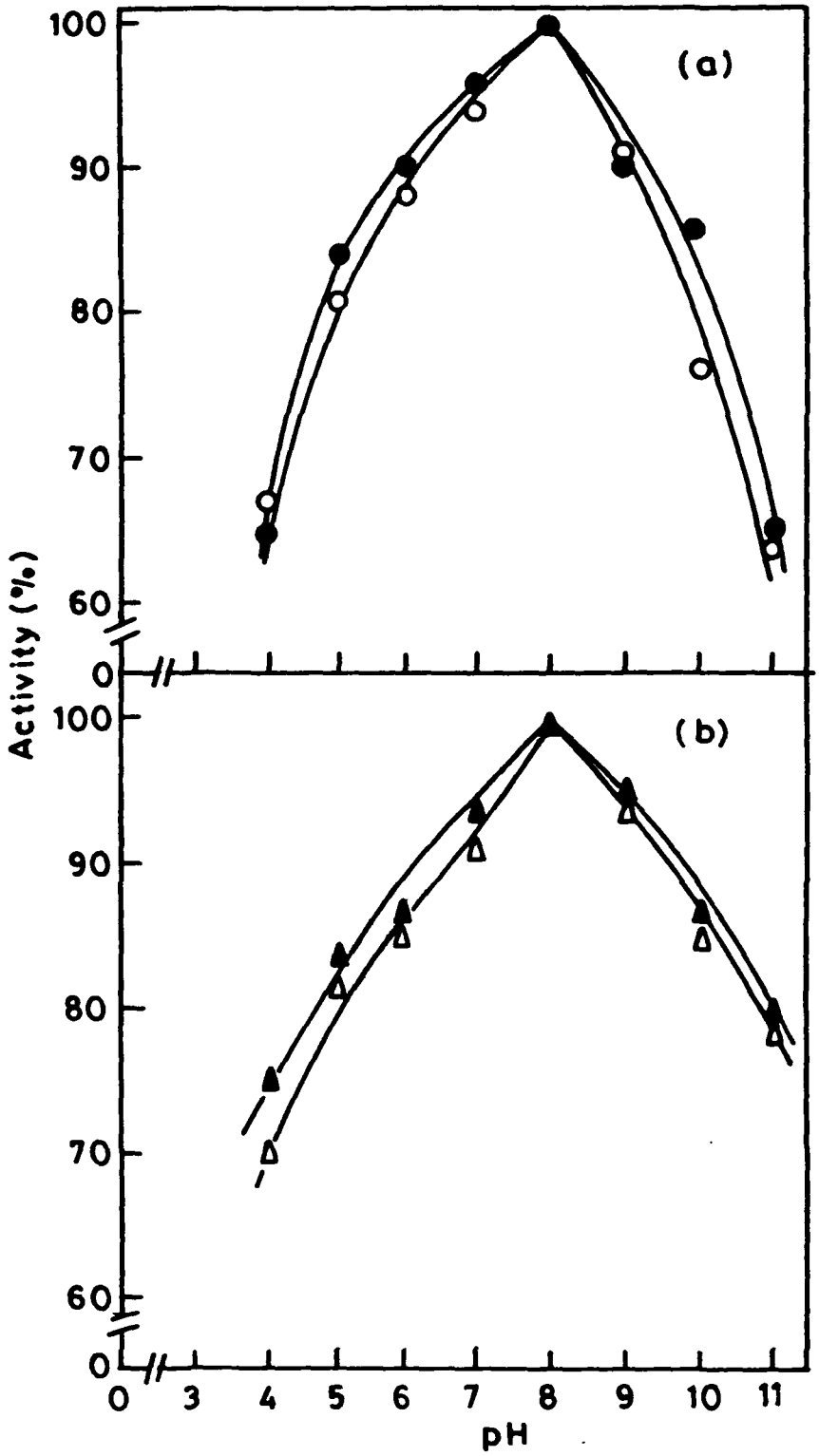

(a)
(b)
100
90
80
70
60
0
Activity (%)
0
30
60
90
120
150
Time (minutes)

Fig. 35 pH dependence of the enzymatic activity of soluble and immobilized papain preparations (H&L).

pH dependence of the enzymatic activity of papain directly immobilized on Seralose-4B with high (a) or low (b) enzyme concentrations, was carried out at 37°C under standard conditions.

(o, Δ) Soluble papain

(●, ▲) Immobilized papain


(a)
(b)
Activity (%)
pH
100
90
80
70
60
0
3
4
5
6
7
8
9
10
11

DISCUSSION

Proteases are group of enzymes which bring about the degradation of protein macromolecules. Due to their industrial, pharmaceutical and medicinal potential, these proteases are focus of attention by many researchers (Guisan et al., 1991; Virnik et al., 1996, Markvicheva et al., 1994). The selection of particular protease for a given purpose depends on the protein to be degraded and on the extent of required degradation. Among the proteases characterized so far, papain is one which can be used multidirectionally including its great and successful potential in the medicine (Neikov et al., 1988; Slosberg et al., 1987; Udod et al., 1987). Papain is used during beer finishing operation as a chill proofing agent to ensure the long term brilliance and colloidial stability of the final product (Wiseman, 1993). Proteases prevent haze formation by partially degrading the proteins that are necessary for the haze formation. Plant enzyme papain is most widely employed chill proofing protease because it catalyzes only a limited break down of the protein. Papain is also considered as meat tenderizer and is widely used in the meat industries (Swanson et al., 1992).

Very recently it has been shown that papain and other proteolytic enzymes seem to act like the lysosomal enzymes that are released in inflammatory disease processes, which mediate inflammation by stimulating the synthesis of prostaglandins. A model has been proposed by introducing the papain in the blood circulation to induce the inflammation which appears to be

a sensitive and relevent for discovering new and effective drugs against inflammation and rheumatoid arthritis (Scott et al., 1994).

The most potential use of papain in medicine includes the inhibition of tumor growth, invasion and metastasis (Bellelli et al., 1990). The therapeutic effect of papain in terms of the release of cytokines has been demonstrated by Desser and Rehberger (1990) on the basis of papain induced production of tumor necrosis factor alpha in human peripheral blood mononuclear cell cultures in a time dependent manner. In another study, papain has also been used in the treatment of acute destructive lactation mastitis. It shortened the period of purification of purulent wounds of the lactating mammary glands from devitalised tissues (Storozhuk et al., 1985).

In view of the industrial, pharmaceutical and medicinal applications of papain, attempts have been made to stabilize the enzyme and to improve its reusability. Kumakura and Kaetsu (1984), have entrapped the papain and other proteolytic enzymes in the polymer matrix. Covalent coupling of papain has also been observed by several workers (Huckel et al., 1996; Zhuang and Butterfield, 1993; Hayashi and Ikada, 1990). Kondo et al (1994a) have immobilized papain on ultrafine silica particles. The preparation was more effective for extractive bioconversions. A physical adsorption of papain followed by crosslinking with glutaraldehyde has also been achieved by Chiou and Beuchat (1986). In a critical study performed by Huckel et al (1996), where

porous zirconia was used as a support to immobilize the papain, the disadvantages of certain immobilization methods were highlighted. The physiosorbed and entrapped biomatrices, however, tend to leak proteins, leading to a loss in the activity with time as well as contamination of the product by the enzyme. In the pharmaceutical applications of the immobilized enzyme the contamination of the product is not acceptable. The covalent coupling of enzymes can lead to a considerable loss of activity due to the influence of coupling conditions. This method may also result in the change of enzyme structure during immobilization step leading to the steric hindrance of the active site of the enzyme (Axen and Ernback, 1971; Goldman et al., 1968). Huckel et al, (1996) have also pointed out the disadvantages of certain supports such as silica and porous glass which are soluble in aquous alkaline media and certain matrices get hydrolyzed in the acidic solutions (Anspach et al., 1989; Weetall, 1969; Hyndman et al., 1992). Aluminium oxides have also been used as support materials but have the disadvantages of adsorbing anions strongly, thus requiring specially optimized surface derivatization chemistries, and tend to be less resistant to grinding or mechanical abuse.

One potential approach in the immobilization of enzymes that does not involve chemical modification and facilitates excellent access of the substrate for bound enzyme is the use of bioaffinity matrices including specific antibody supports. Enzymes immobilized on support precoupled with antibody exhibit

almost full catalytic activity on their respective substrates. The problem in immunoaffinity immobilization of enzyme is the presence of antibody inhibitory towards the enzyme. The non-inhibitory monoclonal antibodies can be conveniently screened from among the spectrum available from various hybridoma clones (Solomon et al., 1984). Antiserum of animals immunized with enzymes may, however, contain inhibitory antibodies (Shami et al., 1989) in which case they may be separated by conventional protein affinity fractionation procedure (Sada et al., 1988). Alternatively, it may be possible to raise non-inhibitory antibodies in the animals by immunizing them with enzyme that have active site shielded with a reasonable large ligand (Fusek et al., 1988; Stovickova et al., 1991).

In order to prevent the formation of active site directed and hence inhibitory antibodies, active site of papain was modified with iodoacetamide and iodoacetic acid before using it as an antigen. The results in Table I show modification of sulfhydryl group. Active sites of ribonuclease and glutamate dehydrogenase have also been chemically modified (Syed et al., 1994; Crestfield et al., 1963). There was complete loss in the catalytic activity of papain after chemical modification but the electrophoretic pattern was comparable with the native papain (Fig. 2) which clearly suggests that the modified preparation of papain has similar charge comparable to the native papain. Modification of cysteine residues of papain and retention of similar

charge on the protein may be due to no alteration in the electrostatic free energy of the protein molecule. Chemical modification of lysine residues of ovalbumin resulted in an increase in the electrostatic free energy of protein molecule and an increase in the 19 net negative charge (Ansari et al., 1975). Gel filtration pattern of native and modified papain was found to be slightly different. When native, iodoacetic acid and iodoacetamide treated papain were subjected to gel chromatography on Sephadex G-100 column, the modified papain preparations were eluted before the native papain (Fig. 3). This suggests that the modification of active site of papain with iodoacetic acid or iodoacetamide causes a conformational change in the enzyme such that it deviates from its globular shape.

The hydrodynamic parameters of native and modified papain as determined from its gel filtration behaviour on a calibrated Sephadex G-100 column suggested a Stoke's radius of 2.65 nm for the native papain and 3.10 nm for the modified papain (Table II). The increase in Stoke's radius of the modified papain further confirms the result of gel filtration that the shape of the modified papain is deviating from its globular nature.

Antiserum of immunized animals raised against the native and modified papain contained precipitating antibodies as evident from Fig. 5, 6 and 7. Antiserum of the modified papain also crossreacted with the native papain (Fig. 8) which indicates that it is recognizing epitopes common to native and

modified enzymes presumably not located at the active site. The antiserum of papain was non-inhibitory and did not bring about any activation of the native enzyme. In other studies 50% activation of invertase in the presence of antibodies has been reported (Jafri et al., 1993). Antisera raised against the pencillinase (Pollock, 1964; Pollock et al., 1967) or ribonuclease (Cinader, 1967) also stimulate the respective antigens. A high titre for the antiserum in the direct binding ELISA (Fig. 9, 10 and 11) indicate that papain was highly immunogenic in rabbits. Rausch et al (1984) have raised polyclonal antibodies against papain and have reported high titre in ELISA and RIA. Considering the non-inhibitory nature, native papain antiserum as well as purified IgG (Fig. 12) were selected for the studies. Purification of immunoglobulin G-fraction will minimize the effect of serum contaminants during precipitation studies. The immunodiffusion shown in Fig. 13 suggest that purification of IgG did not alter the precipitating nature of antipapain antibody and was comparable with that of antiserum.

The results of immunoprecipitation of papain with antipapain antiserum are shown in Table III. The immunoprecipitation of certain enzymes with their respective antiserum have already been reported (Burnet and Schmidt, 1921; Suzuki et al., 1969) but no one has suggested the use of optimum concentration of enzyme for maximum retention of enzyme activity. Attempts have been made in this direction and the η values shown in Table III are 0.75

and 0.96 for the insolubilized preparations(A) and (B) respectively, where two different enzyme concentrations were precipitated with the fixed amount of antipapain antiserum. The 'low' and 'high' antibody to enzyme ratios in these immunocomplexes reflect their nature with low and high η values, respectively. When the higher concentration of papain was used, all antigenic sites of the antibodies were occupied resulting in steric hindrance and more crowding of the enzyme molecules at different sites of the antibodies. It is very likely that in this particular complex enzyme active sites were not freely accessible to the substrate and η value of 0.75 was obtained. This retention of bound enzyme activity was certainly higher than the activity of the bound enzyme retained by other methods (Hayashi et al., 1993b; Goldman et al., 1968). At 'high' antibody to enzyme ratio (preparation B) where low concentration of enzyme was precipitated with the same amount of antiserum as used for preparation (A), the η value of 0.96 was obtained. The higher η value in this preparation suggested the maximum possible accessibility of substrate to the antibody bound enzyme due to optimum amount of enzyme molecules surrounding the antibody structure.

In order to avoid the effect of serum contaminant during immunoprecipitation on the bound enzyme activity, purified IgG was also used for immunoprecipitation of papain. The results of 'low' and 'high' antibody to enzyme ratios (A & B) are shown in Table IV. An identical pattern of bound

enzyme activity comparable to the results of Table III with a slight improved η values of the two preparations was obtained. This suggested the superiority of immunoglobulin G over the antiserum for the precipitation purposes and further minimized the effect of some serum contaminants on enzyme activity. Jafri et al (1993) and Stovickova et al. (1991) have also used the IgG for immunoprecipitation of invertase and trypsin for maximum possible retention of bound enzyme activities. High retention of activity upon insolubilization of trypsin (Stovickova et al., 1991), Carboxypeptidase (Solomon et al., 1986) and Leucine aminopeptidase (Kohno et al., 1986) with their antibodies has also been reported while other proteolytic enzymes aspartate aminopeptidase (Danielsen et al., 1980) and plasminogen activator (Nielson et al., 1982) did not show much retention of activity under similar conditions.

The usefulness of these complexes depends upon the extent of resistance against denaturating conditions such as high temperature, pH, natural denaturants as well as self digestion by autolysis. The stability of most of the proteolytic enzymes such as trypsin in solution is limited as a result of an autodigestion process which can be minimized only by the addition of calcium ions to the solutions (Petro et al., 1995). The stability properties of papain immunocomplexes prepared in our studies have also been determined in order to see relative effectiveness of different immunocomplexes obtained at different concentrations of enzyme loading.

The thermostability of the papain preparations (A) and (B) obtained by the precipitation of papain with antipapain antiserum and isolated IgG was investigated at 75°C for different time intervals. As shown in Fig. 14 (a & b) the immunoprecipitate (A) exhibited a marked increase in thermal stability and retained 75% activity after 2 hours incubation while its soluble counterpart retained only 25% activity. Shami et al (1989) reported the stabilization of enzymes against heat, proteolysis, oxidative stress and organic solvents as a result of complexing with specific monoclonal/polyclonal antibodies. The results of thermostability of immunoprecipitate using preparation (B) and its soluble counterpart are shown in Fig. 15 (a & b). Immunoprecipitate with antiserum as well as IgG of preparation (B) were most stable retaining almost 100% activity after 2 hours incubation at 75°C and at the same time its soluble counterpart was also more stable when compared with the soluble preparation (A). One possible explanation for higher stability achieved in preparation (B) was just because of the prevention of autolysis due to less number of enzyme molecules while at higher concentrations the crowding of enzyme bring about the autolysis which get facilitated at higher temperatures. Most proteolytic enzymes studied in their soluble form were highly susceptible to autolysis and were inactivated at elevated temperatures (Schnapp and Shalitin, 1976; Zhuang and Butterfield, 1992). The proteolytic enzyme concentration used in the studies of Schnapp and Shalitin (1976) as well as by Zhuang and Butterfield (1992) was possibly much higher which resulted in the inactivation

of these enzymes and possibility of autolysis could not be ruled out. Similar explanation of higher thermostability could also be suggested for the preparations of papain and their soluble counterparts incubated at various temperatures for a period of 30 minutes (Fig. 16 and 17 (a & b)). Schnapp and Shalitin (1976) have also studied thermal denaturation of free and immobilized chymotrypsin and have obtained marked stabilization in the immobilized enzyme. The greater stability of the immobilized enzyme is ascribed to diminished autolysis due to their fixation on the support. Concentration dependent immobilization and stabilization of invertase and glucose oxidase has been shown by Iqbal and Saleemuddin (1983) using varying amounts of concanavalin A precoupled to Sepharose matrix. Thermostability of invertase immunocomplex at single highest concentration was also checked by Jafri et al (1993) with a marked stabilization in the complex in comparison to the soluble enzyme. Although there are discrepencies in the literature concerning thermal stability of other enzymes in addition to papain when comparing immobilized to native states (Klibanov, 1979; Zaborsky, 1973), it is generally agreed that papain, whether immobilized or in its native state (Boyer et al., 1960), has activity over a wider temperature range and at more elevated temperatures than do most proteases. From a practical view point, immobilized papain can be utilized with flexibility with regard to operation temperature, depending upon the nature of substrate and product desired.

A common protein denaturant, urea, was applied to assess the effect of denaturant on the active site conformation of soluble and insolubilized papain. Butterfield and Lee (1994) have shown that urea is effective in changing the active site conformation of papain. Even at 1 M urea a change in conformation was dramatic, when compared with the sample without urea. A higher resistance to denaturation induced by 4 M urea was exhibited by our insolubilized preparations (Fig. 18 (a & b)). In these studies also the preparation (B) was more stable in comparison to the preparation (A) (Fig. 19 (a & b)). However, all insolubilized preparations were more resistant to urea denaturation in comparison to the soluble papain. Zhuang and Butterfield (1992, 1993) have also studied the urea inactivation of free and membrane immobilized papain and enhanced stability was observed in their studies. Butterfield and Lee (1994) have suggested that active site cleft, at least the portion near Cys-25 may have a more closed structure upon urea induced denaturation in the soluble and protected papain. Based on the observations obtained in our studies, the active site cleft near Cys-25 could be protected during insolubilization retaining higher enzyme activity upon urea denaturation. The effect was more pronounced in the immunocomplexes of high antibody to enzyme ratio suggesting a better microenvironment for enzyme survival.

There was no significant alteration of pH activity profile of papain as a result of immunoprecipitation (Fig. 20 and 21 (a & b)). There was neither any

shift in the pH optima nor any significant broadening of the pH activity curve. The rise in enzyme activity around pH 4 observed in all preparations of papain may reflect the presence of aspartic acid in the active site cleft of papain. However, at the extreme pH values used in our studies, papain insolubilized at high antibody to enzyme ratio with antiserum/IgG shows higher activity in comparison to the soluble enzyme suggesting the changes in the conformation of some amino acids around the active site of papain. In the studies of Zhuang and Butterfield (1992) where papain was immobilized differently, some preparations maintained highest relative activity while the free papain maintained the lowest. The pH activity profile of papain and other proteolytic enzymes have been reported unaltered upon immobilization (Zhuang and Butterfield, 1993; Hayashi et al., 1993b).

The kinetic parameters such as K_m and Vmax of native and insolubilized papain at 'high' antibody to enzyme ratios were calculated from the Michaelis Menton equation and the values are shown in Table V. There was slight increase in the K_m of antiserum as well as IgG precipitated papain suggesting a decreased affinity with the substrate. This affinity was relatively lower in the preparation where antiserum was used indicating the effect of serum contaminants and also a decreased η value (Table V). Slight change in the Vmax of these preparations was also observed. The K_m values for

immunoprecipitates of invertase as reported by Jafri et al (1993) did not show any increase but a marginal decrease was observed.

The insolubilized preparations (A and B) of papain could be stored at 4°C for 60 days with a minimum loss in enzyme activity as compared to their soluble counterpart (Table VII). The retained activity of preparation (B) after 60 days was 80% which was higher than the preparation (A). This stability of soluble and insolubilized preparations was similar to other studies mentioned so far and could easily be correlated. Hayashi and Ikada (1990) and Hayashi et al (1993a) have also reported the storage stability of papain upon immobilization. These observed high stabilities of papain could be attributed to the prevention of autodigestion and thermal denaturation as a result of fixation of enzyme molecules on polymeric carriers or during the formation of immunocomplexes.

Heavy metal ions such as Cd^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} are inhibitory for papain (Sluyterman, 1967). The inactive complex could be reactivated in the presence of EDTA. In order to compare the effect of these metal ions on soluble and immunocomplex of papain, an experiment was conducted with increasing concentration of Cd^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} in the presence of fixed amount of EDTA. As shown in Table VI at the lower concentration of these metal ions in presence of 9 mM EDTA, complete reactivation of enzyme was observed by both insolubilized papain and its

soluble counterpart. However, as the concentration of metal ions increased from 1.0 mM to 100 mM, the reactivation of enzyme was not so pronounced at higher metal ion concentration for soluble papain suggesting that metal ions are present in excess and are inhibiting the enzyme activity. At 100 mM concentration of Cd^{2+}, 32% activity of soluble and 74% of insolubilized preparation were retained and similar pattern could be observed by other metal ions of the same concentration. The higher retention of activity by insolubilized preparations of papain could possibly be due to the inability of immunocomplex to form an enzyme-metal ion complex which is most effectively formed with soluble enzyme and bring about the loss of enzyme activity. Sluyterman (1967) has shown that soluble papain is reversibly inactivated in the presence of air and low concentration of cysteine but this inactivation is enhanced by Fe^{2+} and Cu^{2+} and is retarded by EDTA.

Preparation of insoluble enzyme-antibody complexes represents one of the mildest and simplest strategy of enzyme immobilization. Despite their high stability, their small particle dimensions and the ability to pack compactly result in very slow flow rates in the column and restrict the usefulness of the immunocomplexes of enzymes in bioreactors. Another strategy adopted for the preparation of highly active immobilized enzyme is based on the binding of enzymes to suitable carriers via monoclonal/polyclonal antibodies that bind to

the enzyme with high affinity without affecting its catalytic activity (Stovickova et al., 1991; Solomon et al., 1986).

So far emphasis has been given on the use of antibody support for suitable immobilization in terms of high retention of bound activity (Ikura et al., 1984; Nolan and Kennedy, 1990). Jafri et al (1993) have used an antibody support for invertase immobilization where enzyme units taken for immobilization were arbitrary resulting in high η value. It is highly essential to use an optimum concentration of enzyme for immobilization not only to minimize the cost of enzyme but also to achieve an improved product with full retention of enzyme activity and better stability. Attempts have been made in this direction and two extreme concentrations of enzyme considered as 'high' (H) and 'low' (L) were loaded on the antiserum as well as IgG bound Seralose-4B. The results summarised in Table VIII and IX clearly indicate the significance of using extreme enzyme concentrations. As evident from Table VIII where 'high' concentration of enzyme was loaded on antiserum/IgG bound matrices low η values were obtained. During several studies of immobilization, it has been observed that the measured activity of the bound enzyme was lower than the actual bound activity (Naoi et al, 1978; Torchillin et al., 1977). Several explanations have been offered to explain this phenomenon including the blocking of active site (Silman and Katchalski, 1966), destruction of native conformation (Manecki and Vogt, 1976) or modification of side chain residues

essential for the conservation of native structure. Evidences have also been presented indicating the importance of the diffusibility of the substrate (Muller and Zwing, 1982). None of these have used an antibody spacer to link the enzyme with a suitable support. In our studies where antibody spacer has been used for 'high' concentration of enzyme loading (Table VIII) and low η values were obtained, the influence of various factors is expected to be high. Theoretically the antibody spacer should minimize certain factors responsible for the loss in enzyme activity and it is very unlikely that a loss in enzyme activity was achieved in our findings which is a clear indication that some factors, very specifically the accessibility of an enzyme to the substrate is the most predominant one exhibiting a low η value. High η values of Table IX, where 'low' papain concentration was used for affinity immobilization, could possibly be due to the radially accessibility of an enzyme to the substrate. It is expected that the problem of steric hindrance gets minimized during relatively low amount of enzyme loading, and most of the bound enzyme is accessible to the substrate resulting in 'high' η values (Table IX).

When thermal stability of different immobilized (H_1, H_2 and L_1, L_2) preparations and their respective soluble counterparts were studied, noticeable finding was observed. Generally, the immobilized papain on different supports as well as coupled by different methods exhibited higher stability in comparison to the native papain (Hayashi and Ikada, 1990; Bhardwaj et al., 1996;

Goldstein et al., 1970). Similar to these findings our four different immobilized papain preparations also retained higher activity in comparison to their soluble counterparts when incubated either at 75°C for different durations (Fig. 23 and 24 (a & b)) or at different temperatures for 30 minutes (Fig. 25 and 26 (a & b)). Among the immobilized preparations stability was higher at the 'low' papain concentration bound to antibody spacer matrix. These findings resemble with the enzyme antibody adducts where no spacer was used but two similar concentrations of papain were precipitated with antiserum/IgG. The similar explanation for these findings could be given as described in the earlier section i.e. the prevention of autodigestion due to the less number of enzyme molecules. The contribution of thermoinactivation during heat treatment was not clear with the data discussed so far. The higher thermostability of 'low' concentration of papain but in the soluble form draw the attention towards the relationship between thermoinactivation and autodigestion (Fig. 24 (a & b)). As it is clearly evident from the figures, the soluble preparations at two different concentrations retain 25% and 80% activity respectively after incubating at 75°C for 2 hrs. This drastic increase in thermostability at 'low' enzyme concentration could not be explained simply on the basis of different thermoinactivation behaviour at two different concentration. Papain is known for its stability against heat and other denaturants in comparison to other proteolytic enzymes (Chiou and Beuchat, 1986) but nothing has been

mentioned about the specific concentration used to check the stability of enzyme. Our findings of different stability at two concentrations clearly indicate that thermoinactivation is a minor factor and autodigestion of papain is most prominent factor at higher enzyme concentration which gets facilitated at higher temperature. When the enzyme molecules are decreased to a greater extent then this process of autodigestion is minimized to the same extent resulting in higher activity even for 2 hrs incubation at 75°C. This explanation is not simply based on the assumption but a critical analysis of the data available so far on papain properties and its immobilization clearly support our results and its appropriate interpretation. When Goldman et al (1968) have used papain concentration (0.75 mg/ml) which was definitly considered as low concentration, and soluble enzyme was incubated at 75°C for 10 minutes, 75% activity was retained suggesting that concentration of enzyme is responsible for preventing the papain inactivation. In an another study Lozano et al (1993) have used a higher papain concentration (20 mg/ml) and soluble papain preparation at 80°C lost almost complete activity during the initial period of incubation (2-3 minutes). This behaviour of concentration dependent inactivation by heat treatment was not observed in case of other enzymes studied. Glucose oxidase in its soluble form lost 90 to 95% activity after incubation at 60°C for 2 hrs at its lowest and highest concentrations of 0.4 mg/ml and 30 mg/ml respectively (Zaborsky and Ogletree, 1974; Iqbal &

Saleemuddin, 1983). The results of thermostability pattern in the immobilized papain preparations shown in Fig. 23 and 24 (a & b) involved the use of antibody spacer to link the enzyme with the support. Higher stability of immobilized papain as well as other enzymes linked to the support via some spacer has also been reported (Zhuang and Butterfield, 1992; Jafri et al., 1993; Solomon et al., 1986; Hayashi and Ikada, 1990). The insertion of spacer between the support and enzyme reduces the disturbance for substrate-enzyme complex formation since the enzyme bound with the spacer is further separated from the support used and might have higher mobility (Manecke and Polakowski, 1981).

All immobilized papain preparations designated as (H_1, H_2 and L_1, L_2) were more resistant to denaturation in 4 M urea when compared to their soluble counterparts (Fig. 27 and 28 (a & b)). Zhuang and Butterfield (1993) studied the effect of denaturants including urea on papain immobilized through a 6-carbon spacer with higher stability. In their studies further stabilization was achieved in the preparation of papain bound by direct immobilization. Butterfield and Lee (1994) have shown the effect of denaturants on the active site conformation and amidase activity of papain in solution. There was no alteration in the pH activity profile of soluble and immobilized papain preparations suggesting the retention of their original pH activity behaviour after immobilization (Fig. 29 and 30 (a & b)). Zhuang and Butterfield (1992)

have obtained similar optimum pH value for soluble and immobilized papain, but at the extreme pH values the papain immobilized without a spacer maintained the highest relative activity. The comparable K_m and Vmax values of native and immobilized papain using an antibody further indicated the free accessibility of enzyme to the substrate (Table X). Stovickova et al (1991) have also shown the unaltered catalytic activity of trypsin by interaction with the antibodies, even in the presence of excess of antibody. The kinetic parameter of free and biospecifically bound trypsin determined by using BAPNA as a substrate remained unaltered.

The usefulness of antibody spacer during immobilization could also be explained by comparing the results of direct immobilization on Seralose-4B. The η values of two papain concentrations immobilized directly on Seralose-4B were lower than the papain immobilized using an antibody spacer (Table XI). It is a clear indication of greater freedom to the enzyme active site while hanging on the solid supports. The stability properties of directly immobilized papain using different concentrations were slightly higher in comparison to the antibody bound matrix (Fig. 32, 33 and 34 (a & b)). This observation might be due to the difficulty of changing the resulting enzyme conformation after binding papain onto the support (Zhuang and Butterfield, 1993). This also suggests that the multipoint attachment in the immobilized papain without spacer stabilize the enzyme through reduction in molecular mobility (Hayashi

and Ikada, 1990). Hayashi et al (1993a) have immobilized papain directly onto the surface of porous poly(vinyl alcohol) beads and have reported higher stabilities of the immobilized enzyme. Papain was covalently immobilized onto the surface of porous zirconia by Huckel et al (1996) and have obtained higher stabilities. Direct immobilization leads to more protein bound to the matrix, but the enzyme is less active in comparison to the spacer linked enzyme.

Binding of enzymes to suitable carriers via antibody support yield highly active immobilized enzymes. The antibody support represents a rather extended spacer separating the enzyme from the carrier. This reduces the disturbance for substrate-enzyme complex formation and the enzymes would undergo less steric interference by the carrier and consequently have more freedom to react even with the high molecular weight substrates. Enzyme immobilization with a spacer gives an enhanced stability/reusability with minimal conformation alterations of the enzyme active site. Moreover, if the enzyme escapes from the support or undergoes some irreversible change, it could be easily replaced by active enzyme by additional affinity immobilization. This method represents a general technique suitable for the preparation of highly active immobilized enzyme preparations for biochemical studies of enzymes naturally bound in the organelle structures.

SUMMARY

Work described in this thesis represents the immobilization of papain using polyclonal antibodies for its considerable industrial and commercial utility. Detailed investigations were made on the nature, catalytic and stability properties of enzyme complexed with antiserum as well as IgG fraction, immobilized on antibody support and directly on cyanogen bromide activated support.

In order to raise noninibitory antibodies against papain and to use them as a support for its immobilization, the active site of papain was modified with iodoacetic acid and iodoacetamide. The electrophoretic pattern of the modified papain remained unaltered and was comparable with the native papain. Gel filteration pattern of native and modified papain was slightly differrent. The antisera raised against native and modified papain was found to have precipitating antibodies as evident from immunodiffusion studies. Antiserum of the modified papain also crossreacted with the native papain suggesting that it recognizes epitopes common to native and modified papain not located at the active site. A high titre for antiserum in the direct binding ELISA was observed indicating that papain is highly immunogenic in rabbits.

In order to eliminate the serum contaminants, IgG was purified from antiserum on DEAE cellulose column. The cross-reactivity of IgG with the

native papain was similar to the antiserum indicating that the purification did not affect the precipitating nature of antipapain antibodies.

A concentration dependent insolubilization of papain with antipapain antiserum has been achieved. Two insolubilized preparations designated as A and B with 'low' and 'high' antibody to enzyme ratios were obtained. Most of the papain activity was retained by these complexes as evident from the high η values. The η value of papain in immunocomplex A was somewhat lower than those of B suggesting some restriction in the accessibility of papain for its substrate due to steric hinderance. Similar set of experiment was repeated with IgG fraction isolated from antipapain antiserum in order to avoid any affect of serum contaminants during precipitation. Slight improvement in the η values has been noted in these preparations.

The thermostability of two papain preparations A and B with antipapain antiserum and isolated IgG was investigated at 75°C for different time intervals. Immunoprecipitates B obtained with antiserum as well as IgG were more stable in comparison to the preparation A. The stability against urea inactivation was also more marked in the preparation B. There was no significant alteration in the pH activity profile of papain as a result of immunoprecipitation. Both immuno-complexes could also be

stored at 4°C for 60 days with a minimum loss in enzyme activity in comparison to their soluble counterpart. The stability of immuno-complexes was also more prominent in the presence of heavy metal ions.

Despite the high stability of enzyme-antibody complexes, their small particle dimension and ability to pack compactly result in very slow flow rates in the column and restrict the usefulness of the immunocomplexes of enzymes in bioreactors. Another strategy adopted for the preparation of highly active immobilized enzyme is based on the binding of enzyme to suitable carriers via monoclonal/polyclonal antibodies that bind to the enzyme with high affinity without affecting its catalytic activity. Two extreme concentrations of papain considered as high (H) and low (L) were loaded on the antiserum as well as IgG bound Seralose-4B. Preparation (L) exhibited high η value in comparison to the preparation (H).

The stability of immobilized papain preparations on Seralose-4B against heat and urea denaturation were also investigated. The immobilized enzyme showed marked increase in stability. The stability of 'low' enzyme bound matrix was relatively high in comparison to the 'high' enzyme bound matrix. A remarkable finding was also noticed with the soluble preparation of papain, a counterpart of 'low' enzyme bound matrix,

in terms of its higher stability. No alterations in the pH activity profile of the immobilized papain was observed. The K_m and Vmax values of all preparations were comparable with each other.

The usefulness of antibody spacer during immobilization could also be explained by comparing the results of direct immobilization on Seralose-4B. The η values of two papain concentrations immobilized directly on Seralose-4B were lower than the papain immobilized using an antibody spacer. The stability properties investigated for directly immobilized papain using different concentrations were slightly higher in comparison to the antibody bound matrix. Direct immobilization leads to more protein bound to the matrix but the enzyme is less active in comparison to the spacer linked enzyme.

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